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*CARBON ISOTOPE FRACTIONATION IN FORMATION OF AMINO ACIDS BY PHOTOSYNTHETIC ORGANISMS**

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When inorganic carbon is converted to living matter during photosynthesis, there is an isotope effect and the carbon-containing compounds of cells have a slightly lower C^{13} concentration than the carbonate and carbon dioxide of the environment.[1] Craig[2] has reviewed the existing data on carbon isotope abundances in nature. Park and Epstein[3, 4] have recently reported on some carbon isotope fractionations in plants grown in the laboratory.

The effect of isotopic substitution on the physicochemical properties of molecules has been well studied for inorganic and nonbiological organic systems.[5] The relative rates of photosynthesis of the C^{13}- and C^{12}-containing molecules are of a similar order of magnitude as observed for reactions involving formation or rupture of carbon bonds. Although it may be difficult to achieve precise understanding of the carbon isotope effect in living systems, the more qualitative and descriptive features are interesting and have already been the subject of geochemical speculation. Thus, Epstein and Silverman[6] have made extensive measurements on petroleum and petroleum fractions. Since marine organisms tend to have a slightly higher concentration of C^{13} than nonmarine forms, carbon isotope measurements may be important to investigations of the origin and migration of petroleum. In a study of a Finnish Precambrian formation, Rankama[7] observed that a problematic fossil, *Coryecium enigmaticum* (probable age $>1.4 \times 10^9$ years) has carbon which is depleted in C^{13} similar to present organic carbon. Rankama's conclusion concerning early life obtained from this evidence has been discussed by Craig.[8]

Detailed study of this effect might produce information relevant to photosynthesis, biosynthesis, and comparative biochemistry. Accordingly, we have investigated carbon isotope fractionation in a variety of photosynthetic and nonphotosynthetic organisms, including some grown in the laboratory under controlled conditions and some that had grown in a natural environment.

A typical experiment consisted of (1) culturing the organisms, (2) extracting the lipides, (3) hydrolyzing the proteins, (4) separating pure amino acids by ion-exchange chromatography, (5) combusting a portion of the amino acids to carbon dioxide, (6) decarboxylating a portion of the amino acids with ninhydrin and purifying the liberated carbon dioxide, and (7) performing an isotopic analysis on the

carbon dioxide fractions with the mass spectrometer. Substantial differences in the isotopic content of the various fractions and the amino acids have been noted.

Experimental.—The major effort was devoted to study of photosynthetic organisms grown on completely inorganic media, the sole source of carbon being carbon dioxide fed to the system. Thus, any isotope fractionation could be related to a known starting material.

Chlorella pyrenoidosa (ATTC 11469) and *Scenedesmus quadricauda* were grown in a solution containing KNO_3, $MgSO_4$, KH_2PO_4, and trace metals[9] and were fed 5 per cent carbon dioxide and 95 per cent air while agitated under constant illumination. *Anacystis nidulens* and *Anabena* sp. were grown on BGM medium consisting of KNO_3, $MgSO_4$, NaCl, $CaCl_2$, K_2HPO_4, and trace metals at pH 8.5. *Euglena gracilis* was grown on a medium at pH 4 containing mineral salts, trace elements, and the necessary amounts of thiamine and vitamin B-12. *Chromatium* sp. was cultured for us by M.D. Kamen and R. Bartsch of Brandeis University on a medium containing NaCl, $NaHCO_3$, $Na_2S_2O_3$, Na_2S, Ca^{++}, Mg^{++}, HPO_4^{--}, and trace metals. *Ochromonas danica* was cultured for us on a completely inorganic medium by Dr. M. B. Allen of the Kaiser Research Foundation. The marine algae *Porphyra*, *Gracilaria*, *Ulva*, and *Illea* were collected for us from the Gulf of Mexico by Dr. P. L. Parker of the University of Texas.

After harvesting, the cells were processed immediately or lyophilized before storage. In the fractionation of algal material, lipides were removed by Soxhlet extraction with acetone, following which the residue was treated with hot trichloroacetic acid for 30 minutes to remove most of the carbohydrate. The residual protein was hydrolyzed with 6 *N* HCl for 18 hours. A very small amount of a mixture of C^{14}-tagged amino acids of high specific activity was added, and the solution was dried *in vacuo*. The amino acids were separated by resin chromatography using an adaptation of methods devised by Stein, Moore, and Hirs.[10, 11] Thus, Dowex-1 was first employed for the isolation of aspartic and glutamic acids. The remaining amino acids were eluted from Dowex-50 8x with 2 *N* HCl followed by 6 *N* HCl. The tagged amino acids aided materially in rapid determination of elution curves. Purity of all eluates was checked by paper chromatography. After drying *in vacuo*, the pure amino acids were split into two fractions. One sample was combusted completely to carbon dioxide for isotopic analysis. The other was treated with an excess of ninhydrin, and the carbon dioxide from the decarboxylation of the amino acids was collected by sweeping it into a trap cooled with liquid nitrogen.

The magnitude of the observed variations in the isotope ratios as compared with a completely random distribution is expressed in units of parts per thousand (per mil) difference in the C^{13}/C^{12} ratio compared with an arbitrary reference substance:

$$\delta C^{13} = \frac{(C^{13}/C^{12})_x - (C^{13}/C^{12})_{ref}}{(C^{13}/C^{12})_{ref}} \times 1000$$

The carbon isotope reference material for each organism studied was the inorganic carbon of the medium in which it grew. For laboratory-cultured algae, this was the tank carbon dioxide used; for marine algae, the inorganic carbon in the ocean. The marine samples were experimentally compared with Solenhofen limestone, National Bureau of Standards isotope reference sample 20, and then normalized to the inorganic carbon in the oceans as given by Craig[12] after taking

into account the relation between the isotope reference standards used by various workers.[13] Precision of isotopic measurements is a crucial problem in experiments involving natural fractionation of isotopes such as carbon. The maximum fractionation of the carbon isotopes during photosynthesis is of the order of $\delta = -28$, while repeated measurements on the same samples of pure organic substances yielded values reproducible to ± 0.1. However, when complicated chemical procedures are employed, much larger experimental errors can be introduced. Such a simple operation as the combustion of organic matter to carbon dioxide could introduce errors due to such factors as incomplete yields and impurities with interfering mass spectra. The isotopic analysis of carbon dioxide obtained by combustion of aliquots of the same sample of the blue-green alga *Anacystis nidulens* gave $\delta = -15.7$, -14.6, -14.9, and -14.7. The standard deviation of these measurements is 0.16.

Studies have been carried out to examine the precision of a number of steps of the procedure. In the over-all reaction for the decarboxylation of α amino acids by ninhydrin at 100°C, there is a rupture of a carbon-to-carbon bond, and a kinetic isotope effect is possible. The ratio of the rates of decarboxylating the C^{12} and C^{13} carboxyl groups of alanine and glutamic acid was studied by carrying out the reaction to only 2 per cent of completion and comparing this CO_2 with that obtained by 100 per cent reaction. The difference observed was very small in the case of alanine. In glutamic acid, the two fractions differed by $\delta = 7.8$. The reproducibility of the total decarboxylation reaction was also studied. A series of six observations on the total decarboxylation of alanine obtained from Fisher Chemical Company yielded $\delta C^{13} = +0.62$, $+2.51$, $+3.08$, $+1.37$, $+1.04$, and $+2.69$. The standard deviation of the determination is 1.14. A similar series devoted to examination of the α carboxyl of glutamic acid yielded $+3.12$, $+1.59$, and $+2.60$, indicating a comparable error.

There is a possibility that carbon isotope fractionation can be produced by incomplete recovery of the fractions of amino acids as they are eluted from ion-exchange resins. This was studied by making an isotope analysis of different "cuts" of amino acid fractions. In the elution of alanine from Dowex-50, 8x, with 2 *N* HCl, very little, if any, isotope fractionation was found. The first 10 per cent had a δC^{13} only $+0.6$ different from the last 10 per cent of alanine eluted from the column. A much larger effect was noted in the elution of glutamic acid from Dowex-1 using 0.1 *M* HAC. The first 8 per cent had $\delta = +24.2$; the last 16 per cent gave $\delta = -29.6$. Thus, substantial errors in the C^{13}/C^{12} ratio can occur from a partial yield of glutamic acid. However, no error need arise from this source if all the molecules of a given substance are eluted and pooled together.

A possible source of variability stems from differences in the physiological behavior of biological material supposedly grown under the same circumstances. Six separate cultures of *Chlorella pyrenoidosa* were grown under conditions as nearly identical as possible. The cells were harvested and combusted to CO_2. The results of the isotopic analyses were $\delta = -23.5$, -23.9, -25.8, -24.6, -22.8, -21.5. The standard deviation for this set of measurements is 1.46, which is larger than that for the combustion and isotopic analysis of algal cells. Analysis of some experiments suggests one major source of variation, the difference in concentration of CO_2 in the growth medium. A culture of *Chlorella pyrenoidosa*,

grown on 0.1 *M* $KHCO_3$ medium at pH 8.3, using only 5 per cent of the total carbon, gave cells with $\delta = -24.4$. Another culture, grown at pH 6.8 with a large excess of CO_2 supplied from a 5 per cent CO_2, 95 per cent air, mixture using a fritted-glass bubbler to saturate the solution, gave $\delta = -25.8$. A third culture, relatively poorly aerated with the same mixture, using a straight glass tubing with large bubbles, gave $\delta = -11.3$.

Duplicate runs on the same culture of *Chlorella pyrenoidosa* were made to estimate the errors in measuring the isotopic composition of algae. The results are shown in Table 1. The algae were grown under conditions of poor CO_2 supply, and the C^{13} content of the cells is not typical.

TABLE 1

INDEPENDENT PROCESSING OF *Chlorella pyrenoidosa*

	Culture 2a	Culture 2b
Tank CO_2	0.0	0.0
Total organic carbon	−11.3	−11.3
Glutamic acid		
Total	−9.9	−8.8
Carboxyl	+9.6	+5.5
Remainder	−14.8	−12.4
Aspartic acid		
Total	−3.5	−1.9
Carboxyl*	+8.7	+6.8
Remainder	−15.7	−10.6
Glycine		
Total	−2.1	−3.4
Carboxyl	−5.0	−3.2
Remainder	+0.8	−3.6
Alanine		
Total	−5.9	−5.8
Carboxyl	+11.9	+10.1
Remainder	−14.8	−13.8

* Both carboxyl groups.

TABLE 2

C^{13} CONTENT OF SOME MAJOR FRACTIONS OF PHOTOSYNTHETIC ORGANISMS

	Total cells	Lipide	Total amino acid	Carboxyl of total amino acid
Chlorella	−18.9	−22.8	−16.0	—
Anacystis	−14.8	−18.9	−13.0	+1.3
Scenedesmus	−11.9	−17.3	−10.1	+0.9
Chromatium	−17.8	−20.6	−17.3	−6.4
Euglena	−16.4	−18.5	−15.4	−9.1
Ochromonas	−19.5	−22.7	−15.6	−5.4
Average	−16.55	−20.13	−14.57	−3.12

Table 2 shows results of measurements of some fractions from a number of algae. Some general statements can be made. The C^{13}/C^{12} ratio of all organisms examined was lower than that of the input carbon. The lipide fractions were even more depleted in C^{13}, while the carboxyl carbons of the amino acids differed relatively little from the original CO_2. In view of the magnitude of probable errors in measurement and other sources of variation, it is not possible from these measurements to be certain that biochemical unity exists among these organisms. On the other hand, the data of Table 3 agree within experimental error with the hypothesis that the pathways involving isotope fractionation are similar in all the organisms studied. Results of examination of

TABLE 3

THE C^{13} CONTENT OF THE AMINO ACIDS IN PHOTOSYNTHETIC MICROORGANISMS (INPUT INORGANIC CARBON 0.0)

Amino acid	*Chlorella pyrenoidosa*	*Anacystis nidulens*	*Scenedesmus quadricauda*	*Chromatium sp.*	*Euglena gracilis*	*Gracilaria sp.*
Glutamic acid						
Total	−18.7	−11.1	−22.6	−15.6	−17.3	−17.2
Carboxyl	− 8.8	+11.7	− 5.6	+ 2.2	− 0.2	− 8.4
Residual	−21.1	−16.7	−26.6	−20.1	−21.5	−19.3
Aspartic acid						
Total	− 6.6	− 9.7	−12.3	−21.8	− 9.6	−14.4
Carboxyl*	2.2	+ 2.8	− 0.8	−15.9	+ 1.0	− 5.3
Residual	−15.6	−22.1	−24.3	−27.6	−20.3	−18.4
Serine						
Total	− 5.7	—	—	− 6.6	− 8.3	−14.1
Carboxyl	2.5	—	—	− 2.9	− 6.1	− 8.8
Residual	− 9.9	—	—	− 8.4	− 9.5	−16.8
Threonine						
Total	−14.5	—	—	—	− 8.6	−14.5
Carboxyl	− 0.5	—	—	—	+ 2.1	− 8.5
Residual	−19.2	—	—	—	−12.2	−16.4
Glycine						
Total	−14.3	−10.0	−14.7	—	−12.8	−10.2
Carboxyl	− 5.3	− 8.8	−11.8	—	−11.6	−12.8
Residual	−23.3	−11.1	−17.5	—	−14.1	− 7.7
Alanine						
Total	−10.3	− 9.8	−18.2	—	−14.3	−15.3
Carboxyl	− 7.3	+ 0.2	−13.5	—	−13.8	−11.5
Residual	−11.8	−14.8	−20.5	—	−14.6	−17.2
Isoleucine						
Total	−19.6	−10.5	−16.1	—	—	−18.8
Carboxyl	− 7.4	− 1.5	−13.1	—	—	−14.0
Residual	−22.0	−12.2	−17.7	—	—	−19.8
Leucine						
Total	−22.7	−17.3	−21.4	−23.5	−23.5	−22.5
Carboxyl	−16.3	−15.0	−17.8	−22.5	−13.6	−24.1
Residual	−26.0	−17.8	−22.2	−23.7	−25.6	−22.1
Lysine						
Total	−17.0	—	—	—	−22.8	—
Carboxyl	2.5	—	—	—	−24.8	—
Residual	−20.9	—	—	—	−22.4	—
Arginine						
Total	−19.2	—	—	—	−15.9	−13.8
Carboxyl	− 2.6	—	—	—	+ 3.6	− 3.6
Residual	−22.6	—	—	—	−19.8	−15.9
Tyrosine						
Total	−19.8	—	—	—	−17.1	−23.0
Carboxyl	4.1	—	—	—	− 3.6	− 8.3
Residual	−22.8	—	—	—	−18.8	−24.8
Phenylalanine						
Total	—	—	—	—	−18.6	−21.2
Carboxyl	—	—	—	—	− 2.2	− 9.4
Residual	—	—	—	—	−20.6	−22.7

* Average of 2 carboxyl groups.

some of the amino acids of a series of organisms are shown in Table 3. In spite of variations among the cultures, there are consistent relationships. The carbons of the carboxyl groups of aspartic and glutamic acids are always markedly heavier than the remainder of these molecules. In all instances, the carboxyl group of leucine is lighter than that of any other amino acid. The average for total glutamic acid of six organisms is $\delta = -17.1$; that of corresponding α carboxyl carbons of glutamic acid is $\delta = +0.2$. In contrast, the average for total leucine is $\delta =$

-21.8, while that for carboxyl of leucine is $\delta = -18.2$. Such substantial differences for the values of the respective carboxyl carbons is indicative of drastic differences in the corresponding pathways of synthesis—a theme that will be discussed more fully later.

In photosynthesis, a large number of chemical events occur simultaneously—synthesis of carbohydrates, proteins, and lipides. *Chlorella* is capable of growing in the dark using glucose as a carbon and energy source. It was instructive to examine isotope fractionation in such an organism without photosynthesis occurring. For comparison, a culture of *Escherichia coli* was also grown using glucose as an energy source.

Chlorella pyrenoidosa growing in the light on an inorganic medium with CO_2 was transferred to a medium of the same inorganic salts with 5 per cent glucose added. In the light, with 5 per cent CO_2 aeration, the alga adapted to glucose. After a good growth rate had been obtained, a small aliquot of the culture was transferred to a new glucose medium, placed in the dark and aerated with CO_2-free air. Just before harvesting the cells, the respired CO_2 was collected by passing the air stream through barium hydroxide solutions. *Escherichia coli* was grown aerobically in a 1-liter glucose-containing culture placed in a sealed 5-liter bottle. A portion of the gas phase was sampled for isotopic analysis of respired CO_2. The glucose used in the preparation of the culture medium was fermented to ethyl alcohol and carbon dioxide by yeast in a nitrogen atmosphere. The evolved CO_2 was swept into barium hydroxide solution by the stream of CO_2-free nitrogen. After a few per cent reaction, the alcohol-water solution was distilled off and oxidized to acetic acid by alkaline permanganate. The acetic acid was distilled off, neutralized, and evaporated to dryness. The resulting sodium acetate was combusted to carbon dioxide. The precipitated $BaCO_3$ was converted to CO_2 by treatment with 100 per cent phosphoric acid. If the average six carbon atoms of glucose are assigned $\delta = 0$, the CO_2 measured -3.4 and the ethyl alcohol measured -2.8. It was also determined that there is only a small isotope effect in the decarboxylation of pyruvate by yeast extract. For the manometric determination of pyruvic, the procedure of Umbreit, Burris, and Stauffer[14] was followed, and the CO_2 evolved after 5 per cent reaction was compared with that evolved after 100 per cent reaction. The ratio of the rates of decarboxylating C^{12} and C^{13} carboxyl groups of pyruvic acid was found to be 1.0020. The simplest explanation of these results is that glucose does not have large differences in the isotope ratios in the individual carbon atoms and that the Embden-Meyerhof reactions do not have a large carbon isotope effect.

The cells of the glucose-grown *Chlorella pyrenoidosa* and *E. coli* were fractionated as described previously and combusted to CO_2. If the average of the carbons in the glucose is assigned $\delta = 0.0$, the results obtained for *Chlorella pyrenoidosa* and *E. coli* were, respectively: total cells, -0.1, -1.9; lipides, -7.0, -7.0; total amino acids, 0.0, -1.8; carboxyl groups of total amino acids, -2.1, 3.8. The values for the individual amino acids are shown in Table 4.

The isotope ratios of the carboxyls of most of the amino acids do not differ greatly from the remainder of the molecules, which in turn have about the same ratio as the input glucose. The striking features are the low C^{13}/C^{12} ratio of lipides and a perhaps surprisingly light value for the carboxyl group of leucine.

One means of examining steps in the fixation of carbon dioxide in photosynthesis

TABLE 4

C^{13} CONTENT OF AMINO ACID IN ORGANISMS GROWN ON GLUCOSE

Amino Acid	*Chlorella pyrenoidosa*		*E. coli*	
	Total	Carboxyl carbon	Total	Carboxyl carbon
Aspartic acid	−2.9	− 2.6	−3.0	− 4.0
Glutamic acid	−0.3	+ 1.9	−2.4	− 1.5
Alanine	+0.5	− 1.7	—	—
Glycine	−5.8	− 4.7	−3.7	− 3.8
Isoleucine	−5.3	− 5.2	−3.7	− 7.5
Leucine	−5.1	−14.0	−3.1	−18.5
Lysine	−0.9	− 3.0	0.0	− 2.2
Serine	−1.6	− 1.6	−5.5	− 4.0
Threonine	−3.2	+ 2.0	−1.0	− 2.7

and the effect of respiration is to study release of this gas from organisms in the dark following exposure to light.

Five liters of a culture containing about 1 gram, dry weight, of *Chlorella pyrenoidosa* was centrifuged and suspended in 200 ml of fresh culture medium. The suspension was placed in the dark, stirred with a magnetic stirrer, and swept with a stream of CO_2-free air. The evolved CO_2 was collected in a cold trap cooled with liquid nitrogen. Periodically, the collected fractions were removed and dried, the volume was measured, and the gas was admitted to the mass spectrometer for isotope analysis. The results of such an experiment are shown in Figure 1. A culture of algae, grown on CO_2 with $\delta = 0$ and having average carbon in the cells of $\delta = -19$, respired CO_2 of $\delta > 0$.

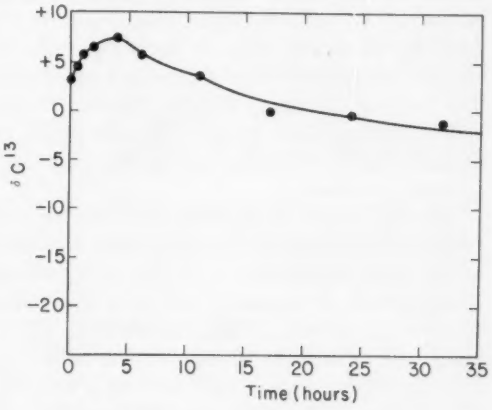

FIG. 1.—C^{13} content of respired CO_2.

The amount of CO_2 collected in the first 24 hours of the experiment was a total of 26.6 ml (STP), representing about 2 per cent of the carbon present. The experiment was followed for several days. The C^{13} content of the respired CO_2 gradually approached that of the total cells. This C^{13}-enriched fraction is probably a residual portion of the feed CO_2 which has been slightly stripped of C^{12} by photosynthetic reactions in the algal cells. The following experiments were performed to give some information on the nature of this pool of material.

About 1 ml of packed, wet cells of *Scenedesmus quadricauda* was treated with 0.1 *M* KHF_2, and the evolved CO_2 was swept into a cold trap. The volume of the gas was 0.9 ml (STP), and it had $\delta = +6.7$. In a similar experiment, when the cells were acidified with 0.1 *M* H_2SO_4, 0.4 ml of CO_2 was collected with $\delta = +5.8$.

It was determined experimentally that when CO_2 and HCO_3^- are brought into isotopic equilibrium, the HCO_3^- is enriched in C^{13}. The equilibrium constant for the reaction

$$C^{13}O_2\ (g) + HC^{12}O_3^-\ (aq) \rightleftharpoons C^{12}O_2\ (g) + HC^{13}O_3^-\ (aq)$$

is 1.0083 at 25°C.

Discussion.—Most of the carbon displayed in Table 3 is depleted in C^{13} compared with the source carbon. In this respect, our observations are in agreement with those of earlier work. The most comparable study is that of Park and Epstein.[3, 4] They examined the C^{13}/C^{12} ratios of marine organisms (green algae, red algae, brown algae, and mixed phytoplankton) and those of four land plants. In all instances, the lipide fraction had relatively less C^{13} than the whole plant, with an average difference of $\delta = 5.7$. Our values displayed in Table 2 agree well with those reported by Park and Epstein on algae with respect to both the total plant and to the lipide fraction.

One experiment performed by Park and Epstein was particularly interesting—a determination of the isotope effect when CO_2 is fixed in the enzymatic carboxylation of ribulose 1,5-diphosphate. Earlier, Calvin and Bassham[15] had shown that this step, which yields two molecules of 3-phosphoglyceric acid, is a principal CO_2 fixation reaction in photosynthesis. Park and Epstein observed that C^{12}-containing molecules reacted 1.017 times as fast as C^{13} molecules, giving rise to a $\delta = -17$.

Our studies of growth of *Chlorella* and *E. coli* on glucose permit us to examine isotopic fractionations in biosynthetic activities other than those involved in making carbohydrate. With the exception of the lipides and the carboxyl carbon of leucine, the ratios do not deviate much from input material. This finding supports the thesis that most of the isotope fractionation in photosynthesis occurs in the carbohydrate-forming process and specifically in the ribulose diphosphate step. The tendency of lipides to have a lower C^{13} concentration than other parts of the cells of the organisms grown on glucose agrees with the findings on photosynthetic organisms and establishes isotope fractionation in the biosynthesis of lipides as a general phenomenon.

The absence of a major difference between carboxyl carbons of aspartic and glutamic acids and the other carbons of most of the other amino acids of the organisms grown heterotrophically contrasts with the findings in photosynthetic growth. The existence of substantial amounts of carbon of different and heavier character in carboxyl carbons of photosynthesizing algae suggests that part of the carbon enters the protoplasm by biochemical pathways other than ribulose diphosphate. Our observations are consistent with a broadly occurring biochemical pattern of CO_2 fixation via the citric acid cycle, and the values obtained in studies of aspartic acid, glutamic acid, and arginine may be rationalized in the light of observations that have been made on a large number of organisms.

In studies of the uptake of $C^{14}O_2$ by *E. coli*, *Torulopsis utilis*, and *Neurospora crassa*, Roberts, Abelson, Cowie, Bolton, and Britten[16] found that CO_2 was incorporated into the carboxyl group of aspartic and glutamic acids. In turn, aspartic acid provided moieties supplying part or all of the carbon chains of α amino adipic acid, isoleucine, lysine, methionine, and threonine, while arginine and proline were related to glutamic acid. Others have demonstrated incorporation of $C^{14}O_2$ into aspartic and glutamic acids in a number of heterotrophic microorganisms and in metazoans including even the vertebrates. Using C^{14}-labeled compounds, Vogel[17] has surveyed biosynthetic activities of a large variety of molds, bacteria,

Euglena, *Chlorella*, and higher plants. The tracers employed were C-3–labeled aspartate, C-4–labeled aspartate, C-1–labeled alanine, and C-2–labeled acetate. He especially studied synthesis of lysine but also observed radioactivity in all members of the aspartic and glutamic families. His findings are in agreement with the existence of a citric acid cycle in the large number of biological materials he has examined. This cycle involves condensation of CO_2 from the medium with pyruvate and subsequent utilization in synthesis, and the CO_2-carbon ultimately appears as the α carboxyl of glutamic acid, as the δ carbon of aspartic acid, and partly in the α carboxyl of aspartic acid. Results from our studies of heterotrophically grown *Chlorella* and *E. coli* show that this biological fixation of CO_2 does not involve a sizable fractionation of carbon isotopes. Our observations on the carbon isotope ratios in photosynthetic organisms are consistent with utilization of the citric acid cycle by these organisms as an independent mechanism for CO_2 fixation. This mechanism explains only part of our observations on carboxyl carbons of amino acids, however.

One important question in the study of photosynthesis is the relative quantitative importance of pathways by which CO_2 is fixed. Calvin has stated that most of the carbon enters via the ribulose diphosphate pathways. His conclusions have been challenged by Warburg,[18] who considers that a pathway involving the α carboxyl carbon of glutamic acid is predominantly important. Our findings coupled with those of Park and Epstein are essentially in accord with the views of Calvin.

Another possible similarity of the synthetic mechanisms of *Chlorella* to those of other organisms is in the formation of arginine. In *E. coli*, it is known that the five-carbon ornithine moiety of arginine is derived from glutamic acid and that the α carboxyl carbon of these two substances has an identical origin. Isotope values indicate that the same situation prevails in *Chlorella*. Thus, the ratios of the corresponding α carboxyl carbons are alike, and those of the remainder of the five-carbon chains are in agreement.

The origin of the guanidine carbon of arginine is an interesting topic. In C^{14} studies of *E. coli* this atom had a high specific activity, and it seemed obvious that the $C^{14}O_2$ tagging this carbon was incorporated directly from the medium and by a path different from that involved in the Krebs cycle.

Arginine was isolated from *Chlorella pyrenoidosa* grown in a mass culture and split by the enzyme arginase. The resultant urea and ornithine were isolated by ion-exchange chromatography and combusted to CO_2 for isotopic analysis. The value for ornithine was $\delta = -15.2$; that of the urea (originally guanadino carbon) was $\delta = +7.1$. The values noted for the guanidino carbon fixed by photosynthetic organisms are particularly significant in the light of this observation. The C^{13}/C^{12} ratio of this carbon differs markedly from that of any carbon of *Chlorella* we have examined. On the other hand, it was noted that this isotope abundance was comparable to that of the metabolic pool of CO_2 found in this organism.

Isotopic fractionation by living matter is not an easy subject of study. In principle, much information about synthetic pathways could be derived from isotopic measurements as illustrated by our speculation regarding the relationship of arginine and glutamic acid. This particular type of question, however, is perhaps most easily studied by using C^{14} tracers. But there is a class of problem that is uniquely accessible only to measurements of natural fractionation. This

can be illustrated by considering a relationship of the lipides to the carbohydrates and proteins. The lipides are derived from carbon fixed initially to ribulose diphosphate. An additional isotope fractionation process must be involved, however, for the lipides are lighter than the carbohydrate or noncarboxyl portion of the proteins. This phenomenon is shown most clearly in the organisms supplied with glucose as the sole carbon source. If the carbon of the lipides is light, conservation laws require that some other portion of carbon is heavier. Or, stated another way, a change in isotope ratios implies the splitting of a population of molecules into two or more classes or streams. Something of this nature must occur after the fixation of the CO_2 by the ribulose diphosphate. Part of the carbon goes to the citric acid cycle, part to carbohydrate, part to lipides, and other portions elsewhere. The isotopic fractionation in the process leading to lipides is probably related to a specific physical chemical step crucially located at the point where a flow of carbon splits into two or more fractions. The isotope measurements then tell us of biosynthetic crossroads and thus potentially reveal a new type of information about the physiology of living systems.

* Read in part before the Academy, November 14, 1960.

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*ON THE INACTIVATION OF TRANSFORMING DNA BY TEMPERATURES BELOW THE MELTING POINT**

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Communicated by Bruno H. Zimm, January 19, 1961

In our studies on the heat inactivation at neutral pH of transforming principle prepared by employing water-saturated phenol as a deproteinizing agent, we have consistently observed exponential heat survival curves at subcritical temperatures, i.e., below the melting point (denaturation).[1] On the other hand, using DNA deproteinized by chloroform–amyl alcohol, we and others[4, 5] observe a survival curve which is concave downward, having a finite initial slope which increases with time of heating.

Since there is a growing interest in the study of heat sensitivity of different markers residing in DNA molecules,[1, 3–6] we wish to describe our experiments showing that the present difference in the behavior under heat is due to the method of preparation of the DNA. The data shown here are for heat-induced loss of the capacity to transform pneumococcus to streptomycin resistance. Similar results were found with three other drug-resistance markers.

The source of the transforming DNA employed in this investigation is a pneumococcal strain recently obtained from Dr. J. Marmur of Harvard University. The strain possesses several unlinked markers, including those for resistance to streptomycin, bryamycin, novobiocin, and erythromycin.

DNA from this strain was prepared by lysing collected cells with desoxycholate and shaking with a chloroform-isoamyl alcohol mixture. The aqueous phase was separated by centrifugation, removed and treated with boiled ribonuclease, following which the chloroform step was repeated, the DNA precipitated with ethanol, and the chloroform and precipitation steps repeated again. The DNA was redissolved in 30 ml of a standard saline-citrate solution and then dialyzed exhaustively against various salt concentrations, ending up in standard saline-citrate. Three weeks later, this preparation was treated with trypsin and chymotrypsin and shaken again with chloroform-isoamyl alcohol, and the aqueous phase was again exhaustively dialyzed against saline-citrate plus 0.01 *M* Tris, pH 8.7. This material behaved reproducibly in the ultracentrifuge and had an $S^{50}_{20,w}$ of 25 Svedbergs. This stock is hereafter referred to as "Sevaged TP."

As a control measure, an aliquot of the above stock solution was shaken manually with an equal volume of water-saturated phenol for 10 minutes; the aqueous phase was separated by centrifugation and precipitated with two volumes of ethanol. The alcohol precipitate was centrifuged and the pellet washed once with 65 per cent ethanol in water to minimize contamination with phenol and taken up in sterile 0.1 *M* phosphate buffer, pH 7.25. The final concentration estimated by the method of Keck[7] was adjusted to 50 μg DNA/ml. This stock is hereinafter designated "Phenoled TP."

A third preparation of DNA, on the heat inactivation of which we have already conducted a series of studies,[1] was extracted from a streptomycin-resistant transformant of pneumococcus strain Rx-1, which originally came from the laboratory

of Dr. Arnold Ravin, University of Rochester, and has been cultured for several years at Yale. A cell suspension was lysed with sodium desoxycholate and shaken with an equal volume of water-saturated phenol for 20 minutes at room temperature, and the two liquid phases were separated by centrifugation. The aqueous supernatant was carefully separated from the denatured protein interphase and the lower phenol phase. The cycle of phenol treatment was repeated once and the aqueous phase bearing the bulk of the DNA was alcohol-precipitated with 2 volumes of ethanol. The precipitate was wound on a glass rod and redissolved in neutral 0.1 *M* phosphate buffer containing 10^{-3} *M* citrate. The resulting solution was again alcohol-precipitated and the precipitate handled in the same manner as described above. The resulting DNA solution was filtered through sterile ultrafine sintered glass filter. The water-clear filtrate was diluted with 0.1 *M* phosphate buffer, pH 7.25, to make a stock solution of 60 μg-DNA/ml (hereinafter designated III-DNA-A). This preparation differed then from the Sevaged and Phenoled TP in these major respects: (1) it had not been treated with RNase or with chloroform-isoamyl alcohol; (2) it had been extracted from a pneumococcus strain which had been cultured for many years separately from the strain from which the Sevaged and Phenoled TP were derived; and (3) it had been put through an ultrafine filter.

Heat inactivation experiments were conducted in 0.1 *M* sodium phosphate buffer, pH 7.25 (measured at room temperature with a Beckman model H pH meter), in a water bath the temperature of which was controlled to within 0.1°. The concentration of DNA during heating was varied according to the experiment but in no case did it exceed 2.0 μg/ml. Details of the procedure of the assay for

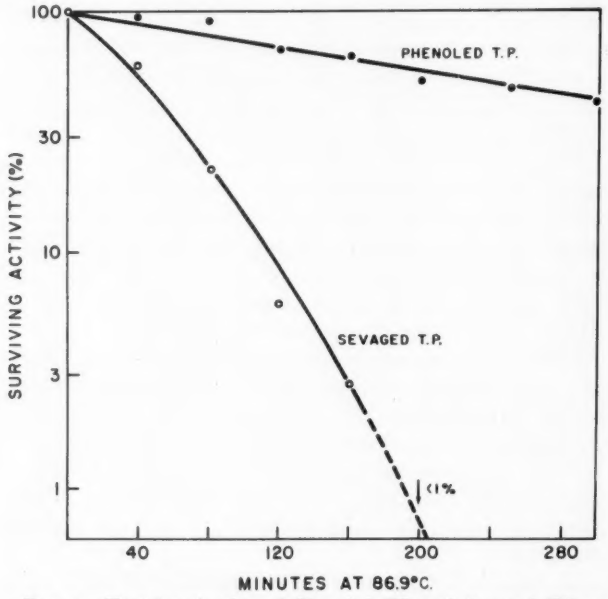

FIG. 1.—Heat inactivation of Phenoled TP and Sevaged TP at 86.9°C in 0.1 *M* sodium phosphate buffer, pH 7.25, at a concentration of 0.5 μg DNA/ml.

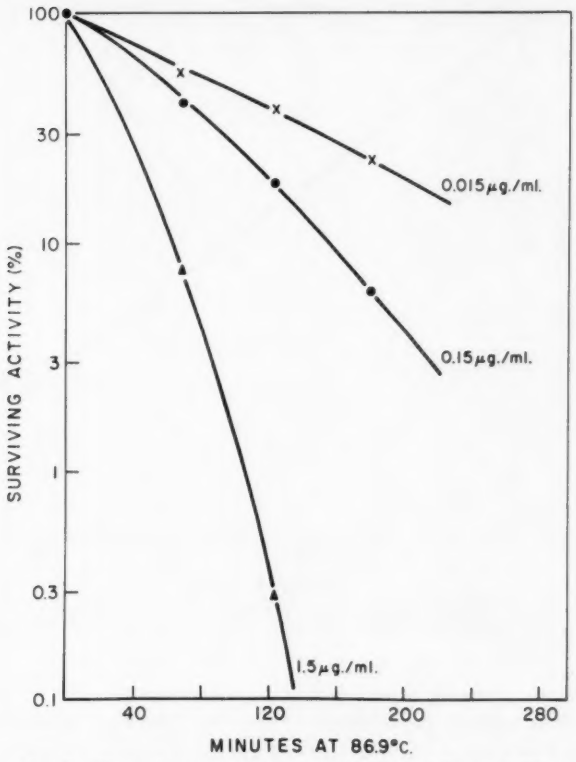

FIG. 2.—Dependence of the inactivation rate of Sevaged TP on concentration of DNA at 86.9°C in 0.1 *M* sodium phosphate buffer, pH 7.25.

transformation are given elsewhere.[8] Three-tenths ml samples, collected at various time intervals, were quick-cooled by transferring into test tubes immersed in a cold-water bath and stored in the refrigerator until assayed. All samples were assayed in a concentration range where the concentration-response was linear.

Figure 1 shows the inactivation curves of the Sevaged TP and Phenoled TP heated simultaneously in the same water bath at 86.9°, both at a concentration of 0.5 μg/ml. It shows a striking contrast in the shape of the survival curves: where the plot of the Sevaged TP is concave downward, that of the Phenoled TP is apparently exponential.

It can be shown, moreover, that the rate of inactivation of the Sevaged TP depends markedly on the concentration of DNA during heating (Fig. 2). The rate for the Phenoled TP on the other hand remains constant over a hundred-fold concentration range, 0.01–1.0 μg DNA/ml (determined in separate experiments). Three samples at 0.015, 0.15, and 1.5 μg Sevaged TP/ml were heated simultaneously in the same water bath at 86.9°. Samples collected at various time intervals were quick-cooled and all diluted to the same concentration of DNA before assaying. While the curvature at 0.015 μg/ml level of concentration has apparently dis-

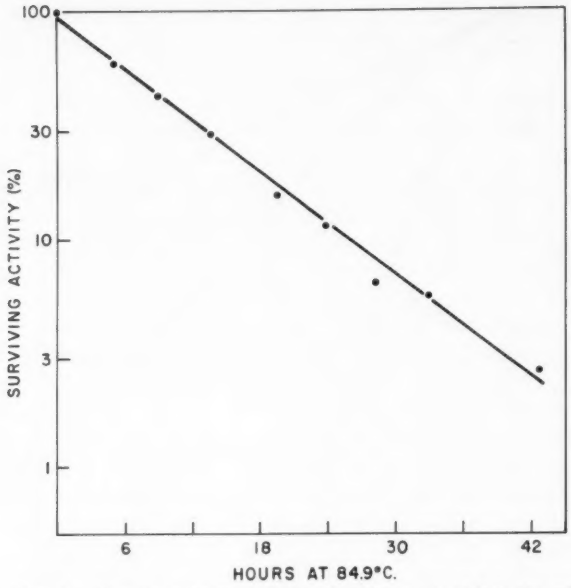

FIG. 3.—Heat inactivation of Phenoled TP at 84.9°C in 0.1 *M* sodium phosphate buffer, pH 7.25, at a concentration of 0.05 μg DNA/ml.

appeared, the rate of inactivation is still 2.3 times greater than for the Phenoled TP.

Figure 3 shows a single inactivation curve of the Phenoled TP at 84.9°. Again, the exponential nature of the inactivation is apparent over a course of 43 hours during which the survivors are reduced to about two per cent of the starting transforming activity. Also, there is no difference at this temperature between four drug-resistance markers (streptomycin, erythromycin, bryamycin, and novobiocin).[1]

Significantly, the rate of inactivation at 86.9° of III-DNA-A ($k = 4.5 \times 10^{-5}$ sec^{-1}) is very similar to that of the Phenoled TP at this temperature ($k = 4.9 \times 10^{-5}$ sec^{-1}). The plot is shown in Figure 4. All survival curves of III-DNA-A, as of Phenoled TP, at temperatures below 87° are exponential for at least 4–5 hours of heating and in the case demonstrated in Figure 4 for a 24-hour course during which 98 per cent of the initial activity is destroyed. Two-component curves become detectable at 88°, a sign of beginning of a second mode of inactivation, i.e., denaturation, which shows a sharp "melting-out" transition as the temperature is raised, with the midpoint for the streptomycin marker occurring at 88.9° for III-DNA-A and 89.4° for the Phenoled TP.[1]

Inasmuch as some residual protein or other material bound to the DNA was suspected as the cause of the complex behavior of the Sevaged TP, its density was examined in a CsCl gradient by comparison to a reference DNA of considerably higher density.[3] The densities of the Sevaged and Phenoled TP's agreed within 0.0005 gm/cm^3, which is probably below the errors of the technique (the Sevaged TP had the greater apparent density). On the other hand, the high salt concentra-

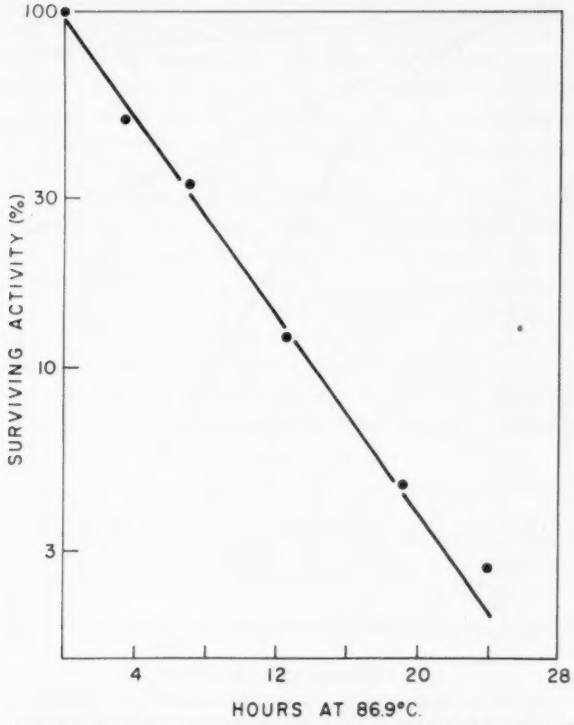

FIG. 4.—Heat inactivation of III-DNA-A at 86.9°C in 0.1 *M* sodium phosphate buffer, pH 7.25 at a concentration of 0.05 μg DNA/ml.

tion is expected to dissociate protein from DNA. From another experiment, we had a fraction of this Sevaged TP which had been collected as a single drop out of a band in a CsCl gradient, so that all unbound contaminating materials should have been removed from the DNA. Figure 5 shows the heat sensitivity of this fraction compared to the others. It is intermediate in stability, suggesting that some but not all of the material has been removed. 0.3 per cent of protein should cause a density decrease of 0.001 gm/cm^3, which was not observed. If this material is protein, the heat sensitivity of TP is a rather sensitive test for its presence.

Discussion.—Heat-induced loss of biological activity of transforming DNA has been ascribed to two mechanisms: chemical degradation (depurination and possibly phosphodiester bond cleavage) and denaturation.[1, 2, 9] At lower temperatures (<87°), where only chemical degradation is discernible, we find that DNA deproteinized by chloroform-isoamyl alcohol is markedly more heat-sensitive than the same DNA further deproteinized by water-saturated phenol. Moreover, in the latter case, the kinetics of inactivation are apparently first-order and independent of DNA concentration, whereas they are complex in the former. The rate dependence on concentration seen in chloroform-isoamyl alcohol–treated DNA suggests a higher-order inactivation process superimposed on the "one-hit" process

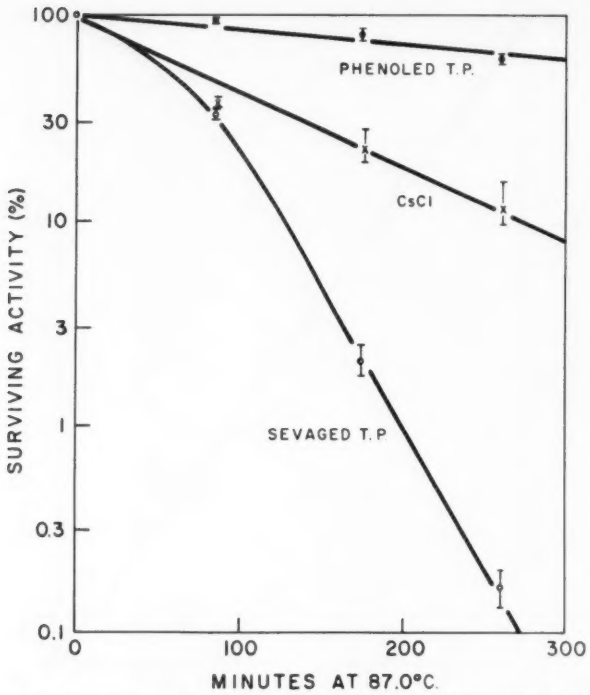

FIG. 5.—Comparative heat sensitivity at 87°C of Phenoled TP, Sevaged TP exposed to CsCl, and Sevaged TP in 0.1 *M* phosphate buffer at pH 7.25.

seen in the phenol-deproteinized material.

The most probable cause of this behavior would be a heat-induced aggregation process due to the presence of an extraneous material, for which protein is the likely candidate. Phenol is thought to be a more efficient deproteinizing agent than chloroform-isoamyl alcohol, whose use must be repeated many times to obtain minimum protein levels. For many purposes in transformation studies, it had not been obvious till now that it was important to carry the purification to the furthest limit, and a few cycles of "sevaging" sufficed. Auxiliary evidence that the degree of protein removal is the key comes from Lerman and Tolmach's work,[2] where the sevag procedure was repeated till no scum remained at the interface. Their curves are reasonably exponential, although their DNA appears to be less stable than our Phenoled TP. The possibility of another material has not been ruled out, but whatever it is, it must be readily removed by phenol and resistant to dialysis and CsCl-gradient separation.

There are numerous other variables in the study of heat inactivation of TP. We have made preliminary investigations of some of these. There is little or no effect of using saline-citrate vs. phosphate buffer as solvent at subcritical temperatures and no very large effect on the melting temperature, T_m. The T_m's also are not greatly affected by the sevag versus phenol procedure, though there may be

small effects here. We find no difference in the sensitivity of four markers at subcritical temperatures, whereas the T_m's are different, as first reported by Roger and Hotchkiss[4] and by Marmur and Doty.[3] No striking effect has yet been found for dependence of subcritical sensitivity on molecular weight, but there is a dependence of the T_m's on molecular weight, which varies for different markers. This will be the subject of a future report.

Summary.—The sensitivity of the transforming activity of a DNA preparation to heating at temperatures below the critical melting temperature depends strongly on the method of preparing the DNA. DNA deproteinized by a few cycles of shaking with chloroform-isoamyl alcohol showed concave-downward, concentration-dependent, survival curves. The same DNA, treated further with a single phenol extraction, exhibited exponential survival curves, which were concentration-independent and more stable and were very similar to the previously observed curves for a phenol preparation from a different strain of pneumococcus. A fraction of the first preparation recovered from a CsCl gradient showed intermediate stability.

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MECHANISMS OF INACTIVATION OF DEOXYRIBONUCLEIC ACIDS BY HEAT

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Communicated January 19, 1961

There has been much interest lately in the structure of the deoxyribonucleic acid (DNA) molecule and the effects of various agents on that structure. In particular, the effect of heat on the properties,[1] structure,[2] and biological activity[3–6] has been the subject of several investigations. Heat is especially interesting in that it leads

to extensive physical changes in the state of the molecule in a critical narrow temperature range. It was recently shown[2, 5] that these changes are at least partly reversible. In addition, heat causes a slow loss of biological activity below the critical temperature[3]; in an accompanying paper[7] it is shown that this loss follows a simple first-order rate law when the DNA is properly prepared. The loss is probably the result of a chemical reaction. Heat further has mutagenic effects in living organisms[8]; these are very likely associated with physical and chemical changes in the chromosomal DNA.

The reported behavior of DNA on heating is in marked contrast to that of ribonucleic acid, which has been studied previously by one of the present authors.[9] In the case of the infectious ribonucleic acid from tobacco mosaic virus, the biological activity is lost by a simple first-order reaction whose rate increases exponentially with temperature.[9] The different behavior of the two kinds of nucleic acid is presumed to reflect the great difference in their configurations, DNA possessing in its native state a rigid, secondarily bonded, double-stranded helical structure,[10] while in RNA, the secondary structure, if any, is certainly less extensive.[9, 11]

The original aim of this investigation was to study in detail the loss of activity of the biologically active DNA, or transforming principle, from *Diplococcus pneumoniae* in order to see to what extent the reportedly complex behavior of this DNA could be related to the simpler behavior characteristic of the ribonucleic acid that one of us had previously studied.[9] To this end, data were taken consisting of the results of quantitative measurements of the transforming activity of the DNA after various heat treatments in aqueous solution. The resulting curves of activity versus time of heating showed so many interesting features that the following rather extensive analysis was undertaken.

Background.—Our general ideas about the effect of heat on DNA are derived from experimental studies such as those already cited and from the statistical mechanical theory that has recently been developed.[12–16] At low temperatures, the Watson-Crick double-stranded structure[10] is the stable form. Imperfections in the structure appear as the temperature is raised; these take the form of occasional sequences of unbonded base-pairs forming open loops. The ends of the chains are natural sites of weakness in the double-stranded structure and extensive opening occurs there also. The opening process leads finally to a catastrophic breakdown of the double-stranded structure at a critical temperature that is identified with the experimental denaturation temperature. At temperatures near but below the critical temperature, the equilibrium state of the molecule consists of a fluctuating alternation of sections of the double-stranded structure and of loop sections of unbonded base-pairs. The fraction of unbonded base-pairs increases rapidly with temperature just below the critical temperature. This change in configuration is accompanied by a change in the optical density of the ultraviolet absorption band at 260 mμ; the latter is frequently taken to be a measure of the fraction of base-pairs bonded, although it is not certain that this is completely justified.[17] Since the chain ends are sites of weakness, the critical temperature should become lower if the chain length becomes too short, although it is not yet known at what length the effect appears. The two strands separate into independent entities above the critical temperature. Since the number of independent particles increases when this occurs, it would be expected that concentration

would have an effect on the critical temperature, but in fact the concentration effect is probably very slight.[16]

A complicating factor is the differing strengths of the bonds in the two types of base-pairs. Marmur and Doty[18] have shown that the critical temperature of a double-stranded molecule containing only adenine-thymine pairs is about 60° lower than that of a guanine-cytosine molecule and that the critical temperatures of molecules with intermediate compositions vary linearly with the composition. Since we have no reason to expect that all the molecules or molecular fragments in a given DNA preparation should have the same composition, we must be prepared to encounter some diversity of critical temperatures within one sample.

The above paragraphs describe the situation at equilibrium, but nonequilibrium effects are also important. The dissociation process is expected to take several seconds or more[19, 20] because of the extensive unwinding that is necessary before the two strands can separate. The rate may be even slower immediately above the critical temperature because the amount of free energy available for driving the unwinding is very small. The reverse process, association, is even more strikingly slow; until recently it was not thought to go at all. However Marmur, Doty *et al.*[2, 5] have recently shown that reassociation will occur if the solutions are held for a sufficiently long time, a period of hours with *D. pneumoniae* DNA, at a temperature somewhat below the critical temperature. This sluggishness is apparently the result of the partial association of various parts of the two strands into improperly matched configurations, which must then be "annealed out" before proper rematching can occur. To distinguish reassociation with reformation of the native double-stranded structure from reassociation into improperly matched configurations, Marmur, Doty *et al.* have coined the term "renaturation" for the former process.

Experimental.—The property that was examined in this study was the transforming activity of the DNA. Cells from a drug-resistant strain of *D. pneumoniae* were lysed and the DNA separated. After having been subjected to various treatments, the ability of the DNA to transform a drug-sensitive strain into a drug-resistant one was assayed quantitatively. The assay was carried out in the linear region of DNA concentrations, where the number of cells transformed is presumed to be proportional to the number of active DNA molecules. Experimental details are given in the previous paper.[7] The results are usually expressed as percentages of the activity of the untreated samples.

Two preparations of DNA were used. The first came from a strain of cells obtained from Dr. J. Marmur, bearing resistance to (among others) the drugs streptomycin, bryamycin, novobiocin, and erythromycin. In the preparation used in this work, the protein was removed from the DNA by extraction with phenol; for this reason, the preparation is called "Phenoled TP." The second preparation came from another strain of cells carrying a streptomycin-resistance marker only; although it too was extracted with phenol, the accidents of laboratory bookkeeping led to its being designated "III-DNA-A." Further details are in the previous paper.[7]

The Collapse Process.—The decay of transforming activity as a function of time at a series of temperatures is shown in Figures 1 and 2 for the various markers tested in the two preparations. One feature is immediately obvious: most of the

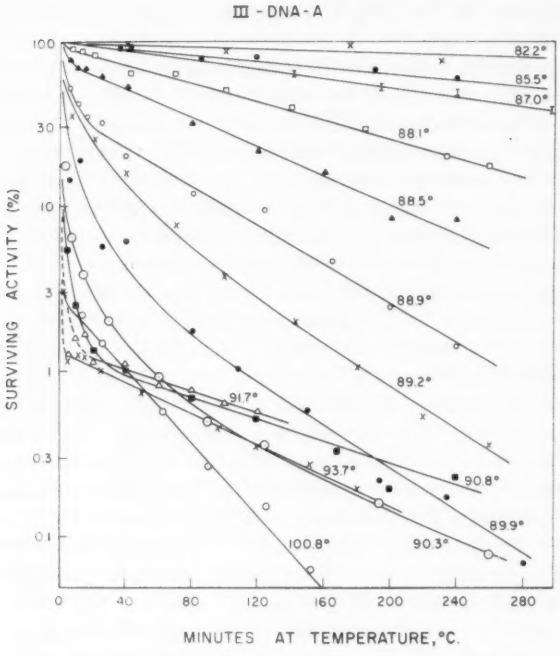

FIG. 1.—Activity for transformation to streptomycin resistance of preparation III-DNA-A after heating for various times at various temperatures. 0.1 *M* sodium phosphate buffer, pH 7.25 at room temperature, DNA concentration between 0.05 and 2.0 μg/ml.

activity curves have two regions; an initial rapid loss of activity followed by a slower decay that is linear on the semilogarithmic scale. Presumably, each of these regions corresponds to its own molecular process with its own characteristic dependence on temperature. For convenience, we name the first process the "collapse process," while the linearity on the semilog scale suggests the name "single-hit process" for the second.

Let us examine the collapse process first. A measure of its extent at any temperature can be obtained by extrapolating the linear part of the curve to zero time and noting the intercept. The latter is presumably the level to which the activity would fall as a result of the collapse process alone if the single-hit process did not occur, and hence, it is a measure of the former.

In Figure 3, the extent of collapse, as measured by the intercepts, is plotted against temperature for all five sets of data. Included also is the optical density curve taken by Marmur and Doty[18] on a similar preparation under similar conditions. The similarity of the six curves is striking, suggesting that the "collapse" in question is indeed the collapse of the native base-paired structure, as has been generally believed.[1, 2, 4, 6]

The data shown in Figure 4 shed further light on the nature of the collapse. These experiments, where the material was returned to a temperature below the

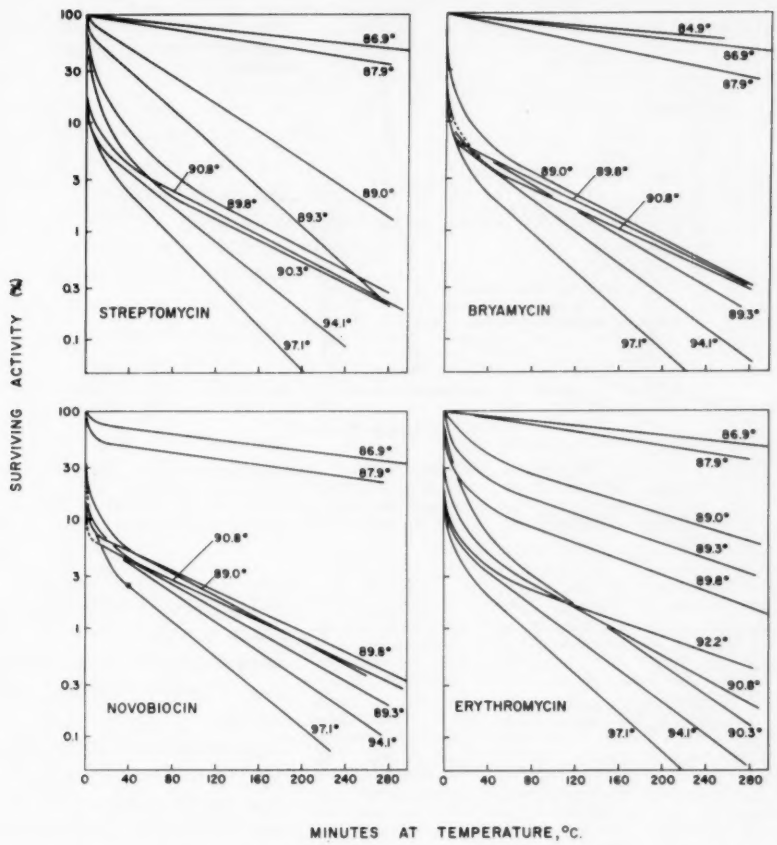

FIG. 2.—Transforming activity of DNA preparation "Phenoled TP" as a function of time and temperature. Four different drug-resistance markers are shown. Same solvent as in Figure 1, DNA concentration between 0.05 and 0.5 μg/ml.

collapse region after a short time above it, show that the process is largely irreversible at the times and temperatures used. This irreversibility suggests, following the considerations outlined in the introduction, that the two strands of the Watson-Crick structure have separated completely.[21]

As was remarked above, the physical separation of the two strands must involve the relative rotation of the ends by as many turns as there are initially in the helix, a rather slow process.[19, 20] The speed of the untwisting should increase with temperature as the free energy available to drive the process increases. This increase is in fact easily seen in the data of Figure 1. Another way the process can be speeded up is by reducing the necessary number of rotations per chain end, for example, by breaking up the strands into smaller pieces before untwisting. In this connection, the data of Figure 5 are interesting. Here, the material was exposed briefly to the degradative enzyme, DNase, before heating. This short

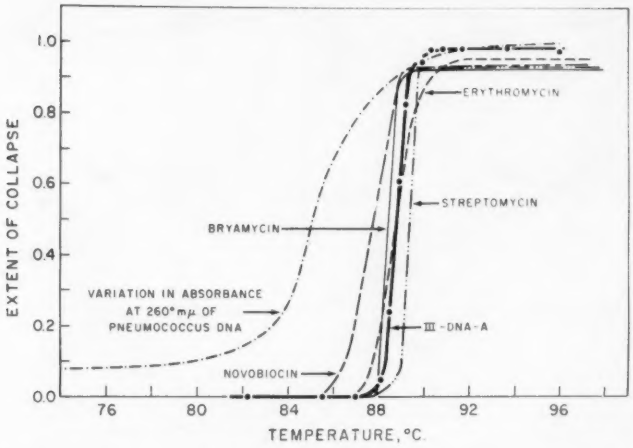

FIG. 3.—"Extent of collapse" plotted against temperature. The straight lines of Figures 1 and 2 were extrapolated to the ordinate; the *loss* of activity at these extrapolated points is taken as the extent of collapse. Also included is the optical density curve of Marmur and Doty.[18]

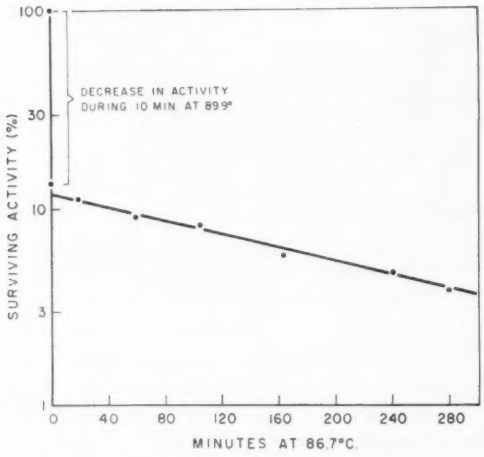

FIG. 4.—Transformation activity of III-DNA-A after initial heating at high temperature and immediate transfer to and holding at a lower temperature. Same solvent as in Figure 1, DNA concentration 2.0 μg/ml at 89.9°, 0.05 μg/ml at 86.7°.

DNase pretreatment has the effect of speeding up the collapse of the surviving activity, in good accord with the expectations.

A remarkable thing about the collapse process is the fact that it never leads to complete loss of activity; a few per cent of the initial activity seem to remain even when the temperature is raised well above that necessary to start the collapse.[21] Several possible explanations present themselves: the activity may be the result of the "renaturation" discovered by Marmur, Doty and others;[2, 5] the sample may contain a small fraction unusually resistant to heat; or the activity may be a true property of denatured DNA.

The possibility of renaturation was tested by heating and cooling at diverse concentrations of DNA. Doty and Marmur [2, 5] found that renaturation was highly concentration-dependent, as would be expected in a reaction involving two

particles. In contrast, we found that changing the concentration of III-DNA-A from 0.05 μg/ml to 10 μg/ml led invariably to between 1 per cent and 1.5 per cent residual activity as determined by the intercept at zero time. Only if the solution were held at 65°C for hours could a different result be obtained, and then only at the higher concentration.[22] This independence of concentration shows that the residual activity does not depend on interactions between the particles and hence rules out renaturation of the kind observed by Doty, Marmur *et al.*

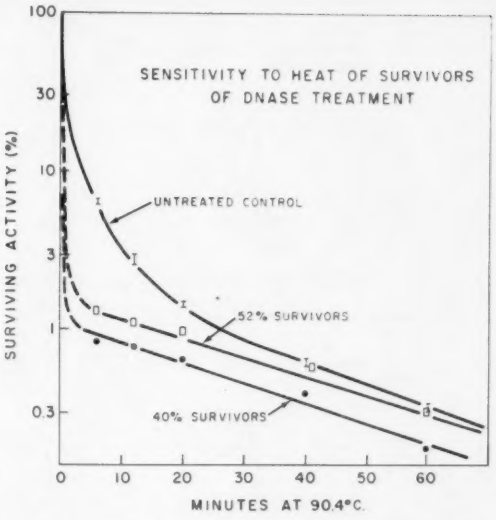

FIG. 5.—Effect of pretreatment with DNase on transformation activity after heating. The ordinate is the per cent of the survivors of the enzyme treatment that also survived the heating. Details: 6.3 μg of III-DNA-A in 0.1 ml of buffer was added to 3.05 ml of the following enzyme solution: 0.01 μg Worthington DNase, 0.4 per cent bovine serum albumin, 0.001 Mg^{++}, physiological saline, and 0.01 *M* phosphate buffer at neutral pH. Samples were incubated at 37° for 7 and for 10 minutes and quickly frozen until the heating experiment. The surviving activity of these two samples before heating was 52% and 40% respectively. After thawing, 0.1 ml of above samples was pipetted into 3.9 ml of 0.1 *M* phosphate in a bath at 90.4°. The control was treated in the same way, except that the incubation at 37° was omitted. The control gave results identical to those of another experiment at 90.4° without enzyme, showing that the DNase was immediately inactivated by the heat.

Decision between the remaining two possibilities is difficult, although good reasons for preferring the third are developed later. If the third is correct, it means that denatured DNA, composed principally of unmatched strands, is effective in transferring information to the receptor cells, though with relatively low efficiency. This idea, though somewhat unorthodox, does not seem to be contrary to any known fact, the details of the transformation process being at present quite incompletely understood. We return to the discussion of this point later.

The Single-Hit Process.—The linearity on a semilog scale of part or all of the inactivation curves is characteristic of a process in which a single randomly occurring event inactivates each genetic marker. We leave aside for the moment the question of the precise chemical nature of this event, referring to it for convenience simply as a "hit."

The remarkable temperature dependence of the rate of this single-hit process, as given by the slope of the inactivation curves, is shown in Figure 6. The process proceeds below the collapse region but slows down very rapidly as the temperature drops. There is a striking similarity between these curves and the dependence of optical density on temperature (Fig. 3). We are led to believe that the site of the "hit" must be at those occasional base pairs that, as the result of thermal fluctuations, are not hydrogen-bonded and to which reference was made in the introduc-

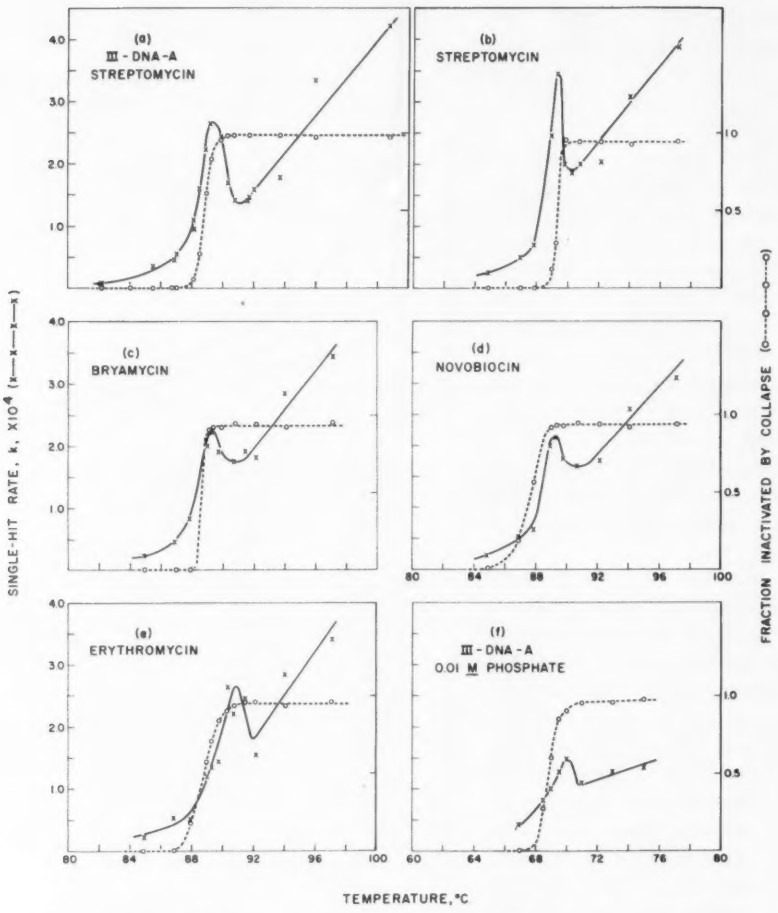

FIG. 6.—Rate of single-hit process, in reciprocal seconds (solid curves) and extent of collapse (dashed curves). Graphs (a) and (f), III-DNA-A in 0.1 *M* (pH 7.25) and 0.01 *M* phosphate (pH 6.6) respectively; graphs (b)–(e), Phenoled TP in 0.1 *M* phosphate.

tion. The rate of the single-hit process thus measures the degree to which the native helical structure is broken down, as does the optical density, but with the difference that the hit is in the narrow region of the genetic marker, whereas the optical density is averaged over the whole sample.

At temperatures immediately above the collapse region, the curve of the single-hit process takes a new course, first dropping precipitously as the collapse becomes complete and then rising smoothly with temperature at a rate quite usual for simple chemical reactions ($Q_{10} = 3.4$, activation energy of about 34 kcal). In order to understand the meaning of this curve, we must discover what the material is that retains its activity after having been heated above the collapse temperature. We still have two hypotheses: there may be a small fraction that is unusually resistant

to heat, or the activity may be a true property of denatured DNA.

Let us begin by examining the first of these alternatives. The data of Figure 6 show that the resistant fraction, while resisting collapse, is nearly as sensitive to the single-hit process as the main fraction. To examine this point further, the experiments shown in Figure 7 were carried out. Here, two samples of DNA were kept for a while at 87°, just below the collapse region, until they retained only 37 per cent and 20 per cent, respectively, of their initial activity. They were then raised to a temperature above the collapse region and the activity followed as a function of time by removing aliquots for assay. This activity, when extrapolated back to the time at which the samples were raised to the higher temperature, gives a measure of the sensitivity of the resistant fraction to heating at 87°, below the collapse temperature of the main fraction. From the straight line, B, in Figure 7, we can obtain the rate constant of the single-hit process operating on the resistant fraction at 87°; it is 2.7×10^{-5} sec^{-1}. This may be compared with the rate constants of 12×10^{-5} at 91.7° and 37×10^{-5} at 100.8° from which the value of 3.4 for Q_{10} was derived; using the same value of Q_{10}, we would expect that the rate constant would be 8.9×10^{-5} at 87°, much greater than observed. We are therefore forced to believe that the single-hit curve for the resistant fraction has a sharp bend in the collapse region of the main fraction.

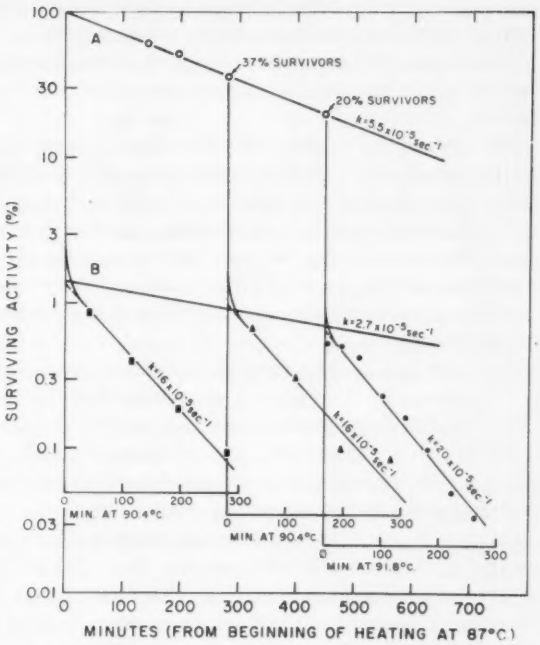

FIG. 7.—Experiments on samples of III-DNA-A heated in 0.1 *M* phosphate for various times at 87° (below collapse temperature) and subsequently at temperatures above the collapse temperature. Samples heated at 87° were assayed for activity at various times (curve A). Two of these and a control were then immediately heated at the higher temperature, as shown. Curve B is the straight line drawn through the extrapolated intercepts of the high-temperature curves. DNA concentration 2.0 μg/ml during 87° heating, 0.05 μg/ml during second heating.

A further test of the idea is given by the inverse experiment to that of Figure 7. The results have already been shown in Figure 4. Here the material was first heated above the collapse region and then its sensitivity to the single-hit process studied by maintaining it at a temperature below the collapse region. We would expect that the inactivation rate would be the same as that of curve B in Figure 7, but we find that it is now considerably larger, 7.0×10^{-5} sec^{-1}, forcing us to con-

clude that a preliminary exposure to high temperature sensitizes the "resistant" fraction to further heating at a lower temperature.

Thus we see that each new experiment demands a new hypothesis, an undesirable state of affairs and one that argues against the correctness of the idea of a resistant fraction in the DNA.

One alternative remains, the idea that the transforming activity that survives heating above the collapse temperature is a true property of denatured DNA. Let us examine from this point of view the facts that have been presented. Since we have already seen that concentration and time of cooling have no effect in the range that we are using, we must believe that the activity does not depend on the interaction of the single DNA strands, or reformation of double strands, that is known to occur to some extent on cooling. It must then be a property of the single strands themselves.

When we look at the data with this idea in mind, one result immediately assumes new significance. In Figure 6, it appears that the drop in the single-hit rate that occurs at the collapse temperature is, within the accuracy of the data, nearly a factor of two.[23] This would correspond nicely to the reduction of the "target" for the single-hit process from two strands just below the collapse temperature to one strand above. Below the collapse temperature, the two strands are still attached and function as a unit on cooling, although many individual base pairs may be unbonded while hot. We must assume, then, that a hit on either strand blocks the activity of both as long as they are attached. Above the collapse temperature, the two strands are separate and remain so on cooling; a hit on one strand blocks this strand alone.

The data of Figure 7 are likewise easily interpreted on this basis. The slope of line B, 2.7×10^{-5}, gives the rate of inactivation of single strands in double molecules which were broken up by heating above the collapse temperature after the inactivation hit occurred. The slope of line A in Figure 7 is the rate of inactivation of the double molecules themselves, and it is just twice, 5.5×10^{-5}, the rate of inactivation of the single constituent strands as given by line B. The results of Figure 7 are thus naturally explained without recourse to additional hypotheses. The same may be said for Figure 4, which is now viewed as giving the rate of inactivation of single-stranded material at 86.7°; it is not expected to have any close relation to the experiment of Figure 7, where the material during the low temperature heating is in the form of double strands with a fraction of the bases unbonded.

Another minor but perplexing observation can also now be explained. In some of the cases shown in Figures 1 and 2, there is a tendency at long times for the activity curves of a temperature in the collapse region to cross those of higher temperatures. We can now say that the lower temperature curves express the inactivation of the double-stranded molecules which inactivate faster than the same material in single-stranded form at a higher temperature. These crossovers are inexplicable on the basis of the resistant fraction hypothesis and furnish the only definite, as opposed to suggestive, evidence against that hypothesis.

To insure that the apparent close relation between the onset of collapse and the rapid rise in the single-hit rate was not the result of an unfortunate coincidence, the series of experiments was repeated with III-DNA-A in 0.01 *M* phosphate buffer. Doty, Marmur *et al.*[2] have reported that the denaturation of DNA occurs between

60° and 70° in this buffer, and we were curious to see if the interrelations found near 90° with 0.1 *M* phosphate would be repeated. The results are summarized in Figure 6(f), and it can be seen that indeed the picture has the same appearance except for the downward shift in the temperatures. Our confidence in the general correctness of interpretation is thus reinforced.

The Nature of the Single Hit.—The question naturally arises: what is the nature of the "hit" that inactivates the transforming activity? Two chemical reactions are known that are likely candidates for the honor, chain scission and depurination. The rate of chain scission of single-stranded DNA was recently determined[2] by measurement of the rate of molecular weight decrease; it amounts to 10^{-6} events per base-pair per minute in 0.01 *M* phosphate at 79°. Zamenhof and Greer[24] have measured the rate of separation of purines from DNA in this buffer; it is about 0.11 per cent per hour at 81° or 1.8×10^{-5} events per base-pair per minute. Clearly, the depurination is much more likely to be the significant event because of its much greater frequency.

We may also estimate the size of the sensitive region. For III-DNA-A at 75° in 0.01 *M* phosphate, we find that the rate of the single-hit process is 8.22×10^{-3} per minute. When this is divided by the frequency of depurination plus chain scission, a total of 1.9×10^{-5} events per base pair per minute, we obtain a quotient of 430 base pairs, which is our estimate of the size of the sensitive region for streptomycin resistance in this material. This is in good agreement with the target molecular weight of 3×10^5, or about 500 nucleotide pairs, which was calculated by Guild and Defilippes for the same marker from data obtained by irradiation with cyclotron particles in the Mev range.[25]

Attempts have also been made by Rosenberg, Sirotnak, and Cavalieri to determine the marker size by measuring the rate of loss of transforming activity as the molecule is broken by sonication or shear.[26] They concluded that the marker was "small" in comparison to the molecules used in that study, which were 1400 base-pairs or more in length. Examination of their published data leads us to believe that there is no essential contradiction between their results and ours, in that a marker size of 430 base-pairs could not be distinguished from one much smaller within the precision of their data on the streptomycin marker.

Heterogeneity.—It is apparent in Figure 3 that there are differences among the markers with respect to the precise temperatures, and the temperature ranges, associated with the collapse process. Similar differences have been observed by other workers,[1-6] and those observed by Marmur and Lane,[5] who worked with the same strain of organisms, are consistent in detail with ours. Hence, it seems unlikely that the differences are the result of random experimental errors. It is tempting to attribute the differences to the presence of different amounts of guanine-cytosine base-pairs in the various markers or possibly to differences in the chain length of the DNA strands bearing the markers. The latter hypothesis could also be invoked to explain the variable sharpness of the collapse transitions as seen in Figure 3. However, these explanations are confronted by a difficulty as soon as the rate curves of the single-hit process are considered, since similar effects would be expected to appear in the rates, while in fact the rates behave otherwise.

We have already presented arguments supporting the idea that the rapid rise in the single-hit rate that occurs just below the collapse temperature is a reflection of

the increasing number of base-pairs that are opened up as a result of thermal excitation. We would expect that those DNA molecules that were especially susceptible to collapse for any of the reasons given in the preceding paragraph would also have an especially large fraction of opened base-pairs, and hence would be especially susceptible to being "hit." However, the experiments show that at any given temperature below the collapse region the inactivation rates of all the markers in both samples of DNA are precisely the same, in apparent contradiction to our explanation of the collapse differences.

We can get around the difficulty by postulating that the separation of the strands that causes the collapse is affected more by the properties of that small most stable region that is the last to come apart than by the average composition of the DNA molecule as a whole. If, for example, the molecule bearing the streptomycin marker in Phenoled TP had a sequence of a few score base-pairs that was particularly rich in the strong guanine-cytosine combination, this sequence would hold the two strands together even when the rest of the molecule was ready to separate. If the molecules bearing the bryamycin marker lacked such an unusually stable sequence, they would come apart at a lower temperature, as in fact is the case. At the same time the gross composition of the two markers themselves could be nearly the same, the fraction of base-pairs opened up at a given temperature likewise the same, and the rate of the single-hit process the same, as is observed.

The different steepnesses of the collapse curves, with some actually crossing others, can now be explained. We have only to postulate that a given unusually stable sequence is not invariably associated with a given marker. This might result from random breakage of the DNA during preparation, the breaks sometimes leaving a given marker attached to one stable sequence, sometimes to another.

Some Unanswered Questions.—A curious difference occurs in the rates of inactivation above the collapse temperature. While the four markers of the Phenoled TP inactivate at about the same rate, the rate of the streptomycin marker in III-DNA-A is only about half as fast. The simplest explanation would be that the size of the marker region in the latter sample is only about half as large as in the former, an idea that would lend interest to a genetic investigation of the relationships between the two strains of cells concerned. But how does one reconcile this explanation with the virtual identity of the inactivation rates of the same two samples below the collapse temperature? More probably, something resulting from the different modes of preparation, perhaps the presence of a large amount of RNA in III-DNA-A, is involved.

The transforming activity of the DNA after having been heated above the collapse temperature, which we have interpreted as activity of single-stranded DNA, is different in the two samples, being about one and a half per cent of the activity of the native form in III-DNA-A and about 8 per cent in the Phenoled TP. This difference also is probably the result of some otherwise inconsequential difference between the two samples, such as molecular weight. It has been shown that transforming activity is highly dependent on the molecular weight, largely as a result of a change in the absorption of the DNA.[26]

Finally, we wish to direct attention again to one part of the interpretation of the sudden drop in the single-hit rate at the collapse temperature, namely, the necessity for assuming that a hit in one strand of the duplex molecule inactivates also the

complementary strand for as long as the two strands are attached but that the complementary strand recovers its activity as soon as the two strands are separated by heat. In other words, a hit in one strand "blocks" the activity of the other only as long as the two strands are attached. We might hypothesize that the cell has two independent mechanisms for using DNA, one for double-stranded DNA and another for single. This seems unlikely. We might alternatively suppose that a hit in one strand produces a lethal mutation, that is, a cell receiving such DNA not only fails to transform but is actually killed. There are many difficulties with this interpretation; to mention one, it is hard to see why the drop in the single-hit rate at the collapse temperature, here considered as the rate of the lethal mutation, should depend on which marker is being assayed, as it obviously does in the experiments of Figure 6. Hence, this hypothesis also seems unlikely. We await the devising of a better explanation.

Summary.—The inactivation by heat of two preparations of the transforming principle of *D. pneumoniae* has been studied quantitatively with the object of elucidating the mechanisms of heat inactivation. These preparations were both deproteinated by the phenol method, which gives preparations showing simple first-order kinetics. Two processes are found, in accordance with previous workers. The first, or "collapse," process is apparently the actual separation of the complementary strands of the DNA and leads to a rapid and kinetically complex loss of from 93 to 98 per cent of the activity. The remaining few per cent of activity is apparently inherent in the denatured DNA and does not depend on "renaturation." The other process, the "single-hit" process, is a first-order reaction that apparently proceeds only at those base-pairs that are open as a result of thermal excitation, and hence its rate increases anomalously fast with temperature in the temperature region just below that at which the collapse occurs. It seems likely that the first-order reaction is the loss of a purine from one of the strands. With this assumption and the known rate of depurination, we estimate the size of the streptomycin resistance marker to be about 430 base-pairs, although this estimate must be considered as very tentative in view of differences between the two samples. The collapse temperature is different with different markers in the same sample, while the dependence of the single-hit process on temperature is nearly the same for all markers as long as the collapse process does not intrude. Following other workers, we attribute the differences in the collapse temperatures to heterogeneities in the guanine-cytosine content of the different markers, but in order to reconcile this idea with the rate data, we must assume that the heterogeneities are very local and that the over-all compositions of all the markers are effectively the same.

One of us (W. G.) gratefully acknowledges the support and encouragement of Professor Ernest C. Pollard during the course of this work.

We are indebted to W. R. Guild for providing the organisms and special facilities and for general advice and encouragement. This work was aided in part by Grant G-6189 from the National Science Foundation. We owe thanks to S. Zamenhof and S. Greer for acquainting us with their depurination results in advance of publication. We would also like to thank Zamenhof, M. Roger, P. Doty, J. Marmur, and J. Lederberg for discussions that were of great help in formulating the interpretations advanced here.

Note added in proof: We would like to call attention to a paper by M. Roger and R. D. Hotchkiss elsewhere in this issue, in which is discussed the works by these investigators in this field.

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[22] In other experiments, some recovery of activity was noted when the solutions were held at 65° for two hours. The exact activity figures were 16% after either 4 or 8 minutes at 92° and 2 hours at 65° at a concentration of 4 μg/ml; 3.5% and 2.5% respectively after 4 or after 8 minutes at 92° and 2 hours at 65° at a concentration of 0.05 μg/ml, in reasonable agreement with the observations of Marmur, Doty *et al.*[3,5] on "renaturation."

[23] We should not forget that it is especially difficult to determine accurately the rate of the single-hit process in this region, since the collapse process itself is prolonged under these conditions and interferes with a clear-cut delineation of the single-hit curve. In fact, it is conceivable that the two processes are not completely independent in this critical temperature range.

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SELECTIVE HEAT INACTIVATION OF PNEUMOCOCCAL TRANSFORMING DEOXYRIBONUCLEATE

By Muriel Roger and Rollin D. Hotchkiss

THE ROCKEFELLER INSTITUTE

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The ability to induce genetic transformations in bacteria by purified specific deoxyribonucleates (DNA) has made possible a study of the dependence of genetic function upon molecular structure. The classic work of Avery, MacLeod, and McCarty,[1] demonstrating high molecular weight DNA as the active material in transformation of pneumococcus, opened the field of chemical genetics to direct experimental approach. That transforming DNA is inactivated by treatment with various agents such as deoxyribonuclease, heat, and dilute acid was originally shown by these authors. Since that time, a number of studies have been undertaken, looking into a greater variety of inactivating agents as well as some of the more quantitative aspects of the problem. A relatively sharp temperature threshold for the heat denaturation of various physical properties of DNA preparations has been reported,[2–5] and the threshold temperature at which biological activity of *Hemophilus influenzae* DNA begins to fall has been correlated with its viscosity decline.[2] It has also been shown by Zamenhof *et al.*[6, 7] that of a variety of different inactivating treatments, UV irradiation, and heat showed specificity in inactivating one genetic marker carried by a DNA preparation more than another. More recently, Lerman and Tolmach[8] have studied heat inactivation of P^{32}-labeled pneumococcal DNA and observed two components in the inactivation curves, which they attributed to two inactivating processes.

We have been studying the heat inactivation of pneumococcal DNA (P-DNA) for the past four years and have found that inactivation by exposure to elevated temperatures can result in two distinctly different selective inactivation patterns as a consequence of two different types of molecular change. The two inactivating processes may be effectively separated by proper choice of the conditions for heating, but separation also seems to require a certain intactness of the starting material. We shall attempt to show here that heat denaturation of P-DNA can be regulated to produce either disorganization at different small sites within DNA molecules or, alternatively, selective damage of entire molecules.

Critical Inactivation Temperatures.—The first type of inactivation we will describe is that obtained when a dilute solution of P-DNA is heated at different temperatures for short periods of time. As seen in Figure 1, the very sharp transition between an active and essentially inactive state of the DNA takes place at distinctly different temperatures for the various genetic markers associated with a single DNA preparation. (Throughout this article, the convenient term "marker activity" is used to refer to the quantitatively determined transforming activity with respect to a specific (drug-resistance) marker.) The P-DNA preparation used in this experiment was isolated from a multiply marked strain of pneumococcus, resistant to five different drugs. The P-DNA was prepared by the method usually employed in this laboratory,[9] in which the principal steps are deproteinization by the chloroform-gel method and alcohol precipitation. Small aliquots of a dilute

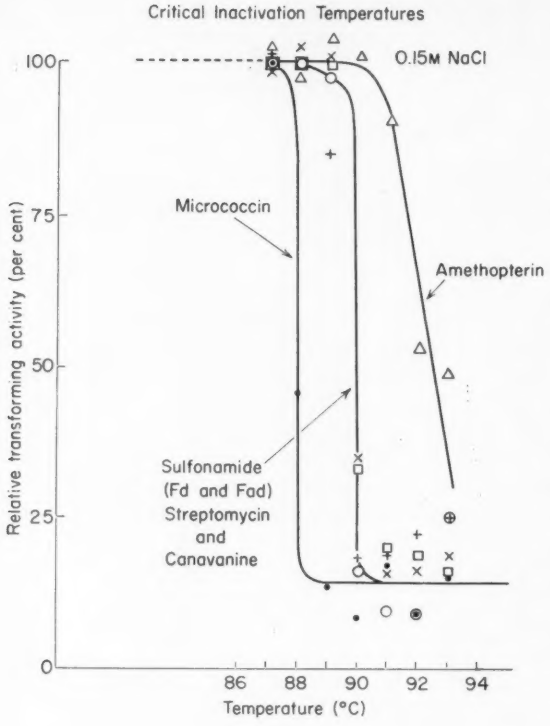


FIG. 1.—30 μg./ml P-DNA dissolved in 0.15 *M* NaCl. Relative transforming activity measured at plateau concentrations by the method described by Hotchkiss.[9] Time of heating 15 min. Symbols: (•) micrococcin, (×) streptomycin, (+) sulfonamide (Fd), (○) sulfonamide (Fad), (□) canavanine, (△) amethopterin resistance marker activities.

solution of the P-DNA in 0.15 *M* NaCl were heated for 15 min at the temperatures indicated.

In this experiment, as in most that will be presented, temperature was maintained constant by use of refluxing mixtures of ethanol-water or methanol-water to give a graded series of different temperatures, constant within 0.1°C. Tubes containing the P-DNA solutions were immersed in the reflux baths for the specified period of time.

The inactivating temperatures are quite high (in 0.15 *M* NaCl, 88° to 93°C), and inactivation of any given marker is a critical function of the temperature. For example, at 87° no detectable loss in activity is seen for any of the five drug-resistance markers. At 88°, micrococcin activity (K) has essentially disappeared, while the other four markers reflect no detectable loss. Two degrees higher, at 90°, sulfonamide (F), streptomycin (S), and canavanine (C) activities fall sharply. The amethopterin (A) marker barely begins to lose its activity one degree higher still, at 91°, at which temperature the other four markers are all essentially inactive. For a given set of conditions, these temperatures are fully reproducible, each marker being associated with a characteristic critical inactivation temperature (T_c). The critical temperatures vary markedly with ionic strength and moderately with small changes in pH, as will be seen, but the order and separation of the T_c's for the individual markers remain the same. Experiments with a number of different P-DNA preparations, derived from the same original strain and carrying different combinations of the markers shown here, have demonstrated that in a defined environment, the T_c's are reproducible for each marker.

The selective inactivation of the various genetic markers carried by the DNA, as seen in Figure 1, is not a difference of degree, as might be caused by differential inactivation rates. Rather, the curves suggest an all-or-none type of inactivating

process, to be discussed in more detail later. That the inactivation is very rapid for all markers is seen in Figure 2. In this early experiment, it was demonstrated that within five minutes heating above their critical temperatures, the micrococcin, sulfonamide, and streptomycin activities were each lowered to a value of about 10 per cent and remained constant at that level for at least 25 min duration. It was on the basis of experiments such as this one that the choice of a 10- or 15-min heating time was adopted in obtaining all activity-temperature profiles. The right-hand curve in Figure 2 was obtained at a ten-fold lower salt concentration (0.015 *M* NaCl) for which the T_c's are below 78°. Not only is the rate of inactivation still very rapid but the residual constant activity of 10 per cent is the same as that obtained at the higher salt concentration. Similar results have been obtained a number of times. The low but appreciable residual activities which appear in all activity-temperature profiles we have obtained are independent of the temperature (that is, above the T_c) and independent of ionic strength. The precise value of the residual activity is dependent upon other factors which are, however, maintained constant within a given series of experiments.

The constancy of the unaltered activity before the critical temperatures are attained and the constancy of the residual low activity after critical inactivation are more clearly shown in Figure 3, which covers a broader

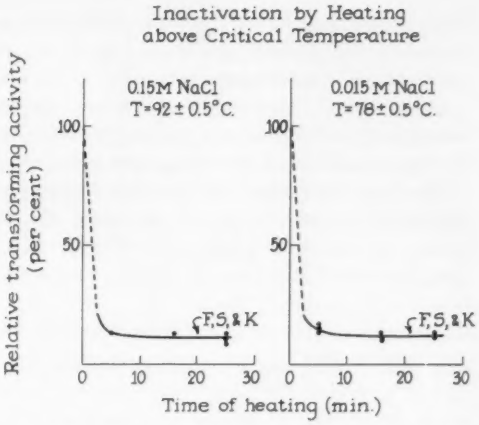


FIG. 2.—50 μg/ml P-DNA dissolved in 0.15 *M* NaCl or 0.015 *M* NaCl. Relative transforming activity determined as described in Figure 1.

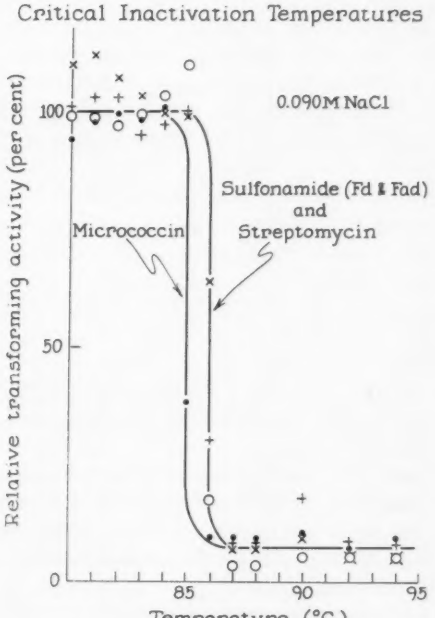


FIG. 3.—50 μg/ml P-DNA dissolved in 0.090 *M* NaCl. Symbols and determination of relative transforming activity as described in Figure 1.

range of temperatures. The salt concentration has been reduced to 0.09 M NaCl, compared with 0.15 M in Figure 1, and, as a result, the inactivation temperatures were lowered three degrees (from 88° to 85° for K). However, a one-degree differential in the T_c's for the three markers carried by the DNA preparation is still maintained. The values of the residual activities show no discernible differences for the three markers, all remaining just below the 10 per cent level.

The activity-temperature profiles of Figures 1 and 3 were obtained by heating unbuffered saline solutions of measured pH, varying between 6.0 and 6.2. Data obtained in 0.14 M saline buffered with 0.02 M potassium phosphate at pH 6.8 are represented in Figure 4. In the buffered saline, the four-degree difference be-

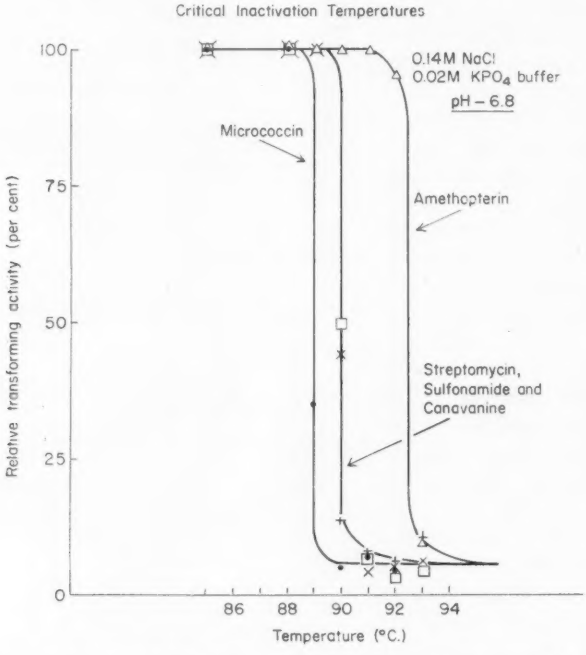


FIG. 4.—30 μg/ml P-DNA dissolved in buffered saline at pH 6.8. Solutions cooled in ice bath. Symbols and determination of relative transforming activity as described in Figure 1.

tween micrococcin and amethopterin inactivation is even more sharply delineated than before. The addition of 0.02 M potassium phosphate has produced a shift throughout of about 1° toward higher temperatures. This we know to be attributable in part to the increased ionic strength and in part to the increase in pH toward neutrality.

Nature of the critical inactivation: With the experimental observation of discrete inactivation temperatures of individual genetic markers firmly established, it becomes appropriate to discuss the significance of these curves in terms of molecular changes. Doty *et al.*[10] have discussed the observed critical changes in certain

optical properties of solutions of calf thymus DNA in terms of a second-order phase transition between ordered helical molecular configurations and more random, coil-like configurations. The optical properties followed were rotatory dispersion and ultraviolet absorption increment.[5] Physical proof that such transitions were actually a result of configurational changes came from previously observed light-scattering data. It had been shown by Rice and Doty[3] that heating of calf thymus DNA for short periods at temperatures between 90° and 100°C produced no change in molecular weight but did result in an appreciable decrease in the average molecular length. Such a change could only be explained by a transition from a highly extended molecular configuration to one that was much more compact. The observation of a ten-fold decrease in intrinsic viscosity accompanying heat denaturation (which had also been reported earlier by others[2, 5]) could now be interpreted as a purely physical, order-disorder denaturation, rather than one involving cleavage of primary chemical bonds to produce degradation products. Thus, both the viscosity and light-scattering data, as well as the increment in ultraviolet absorption, could be explained completely by the nearly simultaneous disruption of the hydrogen-bonded structure of the DNA.

We may associate the observed activity-temperature profiles for P-DNA with just such a helix-random coil transition on several grounds: (1) the critical nature of the transition as a function of temperature, (2) the extreme rapidity of the reaction, (3) the constant residual level of activity, and (4) the light-scattering data we have obtained with both intact and heated P-DNA. The details of these data will be published elsewhere, but certain of the observations are pertinent here. Heating a solution of P-DNA above the critical temperature in a light-scattering cell decreases the effective molecular length from 9,000 Å to something less than 1,000 Å. This effect is similar to that obtained by Rice and Doty[3] for calf thymus DNA; however, the light-scattering data for intact pneumococcal DNA are quite different from those seen by these authors (as well as ourselves) with the thymus DNA. Specifically, whereas the molecular weight of thymus DNA was determined by these authors as 8 million, both before and after heating, intact P-DNA has a minimum molecular weight of 4 million. From the angular distribution of the scattered light for intact calf thymus DNA, it was concluded that the molecular configuration most closely resembles that of a polydisperse, highly extended, random coil. Intact P-DNA, based upon the same considerations, appears to be composed of rod-shaped molecules, showing no indications of polydispersity. Since the P-DNA is rodlike and extremely long, the molecular weight of 4 million determined by light scattering would tend to be an underestimate. The molecular weight and length of the heat-collapsed P-DNA molecule may, however, be unambiguously set at 4 million and 1,000 Å. The decrease in length accompanying heat denaturation brings the crucial ratio of molecular length/wave length of incident light closer to a value for which a reliable zero angle scattering extrapolation may be obtained. The residual activity associated with this heat-collapsed DNA was 10 per cent of that for the intact material, as in the other experiments reported here. We believe critical heat inactivation of P-DNA may therefore be confidently associated with configurational denaturation, in contrast with another type of heat inactivation which will be described below.

Molecular basis of the structural change: Selective collapse of individual mole-

cules within a DNA preparation would then seem to account for the critical inactivation seen in Figures 1, 3, and 4. That DNA from a single species may contain a population of genetically distinguishable, if chemically similar, molecules was first indicated when DNA obtained from multiply-marked strains of drug-resistant encapsulated pneumococci was shown to transfer its individual characters independently of each other.[11] The incidence of cells doubly transformed to any two traits carried by the DNA was always less than that expected from random coincidence (with the exception of the few cases which exhibit true genetic linkage), and it was concluded that transforming DNA in solution behaves like a collection of discrete units transferring single genetic characters. We sought demonstrable differences between these discrete units for this reason.

The chemical basis for the genetic heterogeneity has been assumed to derive from differences in nucleotide sequences. Our first complete activity–critical-temperature profiles were available at the beginning of 1959 and indicated that we were observing a resolution of the individual molecules within the P-DNA population as measured by a dramatic separation of biological activities. In exchanges of information with Drs. Doty and Marmur shortly after that, we learned of the important correlation observed by them[10] and by Meselson[12] between certain physical properties and the overall base composition of DNA preparations. It seemed reasonable that this kind of heterogeneity was involved in the separation of inactivation temperatures we had found. The content of guanine and cytosine, which are believed to be more strongly hydrogen-bonded than adenine and thymine, appears directly correlated with a higher buoyant density (in cesium chloride solution) and with higher temperatures of denaturation (UV absorption increment) in DNA preparations with different overall base composition prepared from different species.

In extending this hypothesis, the spread seen in the absorbance-temperature curves for a particular DNA preparation was taken by Doty *et al.*[10] as a measure of distribution of overall guanine content of *individual molecules* within a preparation. They found P-DNA to show a temperature spread of approximately 4° above and below the mean denaturation temperature of 85°C as measured by UV absorption increment. Our range of 4° between micrococcin and amethopterin inactivation (from 89 to 93°) occurs at significantly higher temperatures than this. On this basis, we would have to suppose that the DNA molecules bearing the amethopterin marker are so much richer in guanine-cytosine than the micrococcin molecules that there is no overlapping in their heat stability. An alternative hypothesis would be that localized fluctuations in base distribution (local guanine-poor regions) might produce critical differences in the heat stability of different molecules without necessarily involving differences in overall base composition.

No current hypothesis leads to a quantitative prediction of the dispersion of "melting" temperatures to be expected within a homogeneous class of identical DNA molecules. The spread in the absorbance-temperature curve of a pure adenine-thymine copolymer was seen to be about 3°, and was taken as an empirical minimum.[10] That the spread in each individual activity-temperature curve for a single P-DNA transformation marker is extremely narrow can be well seen from Figure 5. Even with temperature increments as small as 0.2–0.3° apart, in this experiment only one marker was caught between full activity and the residual in-

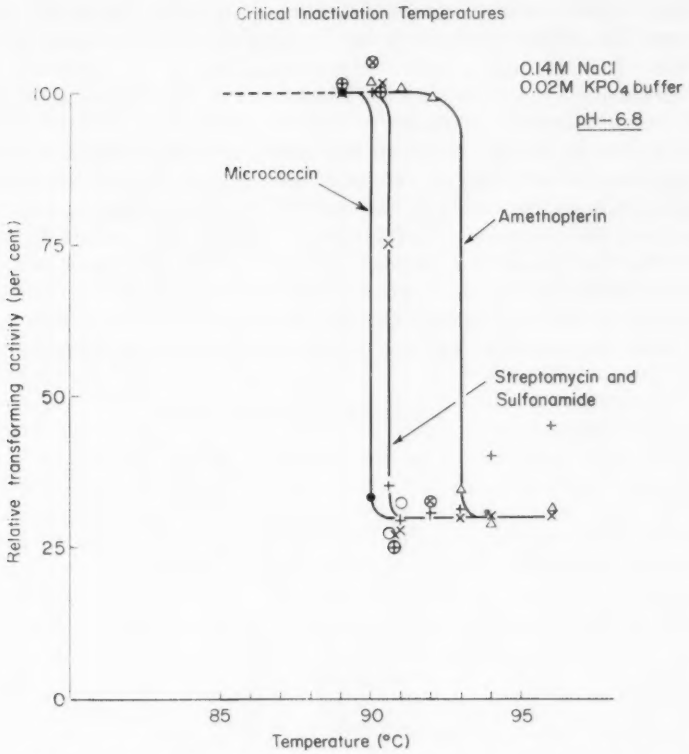


FIG. 5.—30 μg/ml P-DNA dissolved in buffered saline at pH 6.8. Solutions cooled at room temperature. Symbols and determination of relative transforming activity as described in Figure 1.

activated state. The spread is about ±0.2° around the mean of 90.6 (for F and S markers at this ionic strength), i.e., about one-tenth of the total spread we have observed with as few as five markers. This sharpness of inactivation in individual briefly heated samples is supported in other experiments, and has been so indicated in other Figures (esp. 1, 3, and 4). It is clear that such results indicate a homogeneity within individual classes of molecules with respect to some structural feature (whether overall or local compositional differences) far greater than that comprised in the total molecular population.

Certain differences in temperature resistance of genetic markers have been reported[13] as support for the structural hypothesis already mentioned. There are some contrasts in our results which it may be important to point out. We find characteristic inactivation temperatures at which discrete rearrangements occur very rapidly, whereas the results of Marmur and Lane principally indicate different extents of inactivation spreading over a 2–5° range. Thus, in their temperature curves, all three markers have suffered 40 per cent inactivation at very nearly the same temperature (87°), and at no point is an active species seen coexisting with an inactive one. We believe that their inactivation curves show minimal dif-

ferentiation of mean inactivation temperatures and show broad ranges for inactivation of individual markers because of the extended duration of the heating period in which the DNA is being brought to the critical temperature. As will be shown later, such conditions promote appreciable inactivation of a different sort, involving progressive and cumulative damage.

Large deviations in pH away from neutrality lower the denaturation and inactivation temperature of DNA. We find that smaller variations within one pH unit above or below neutrality do not shift most critical temperatures, but can broaden some activity-temperature profiles. When DNA solutions buffered at pH 6.0 rather than 6.8 are heated, there is a spread in the amethopterin inactivation curve of about 2° (Fig. 6) with a resultant decrease of about 2° in the mean T_c. There is no sign of either effect for either sulfonamide or streptomycin markers. A broadened amethopterin inactivation was also obtained in unbuffered saline

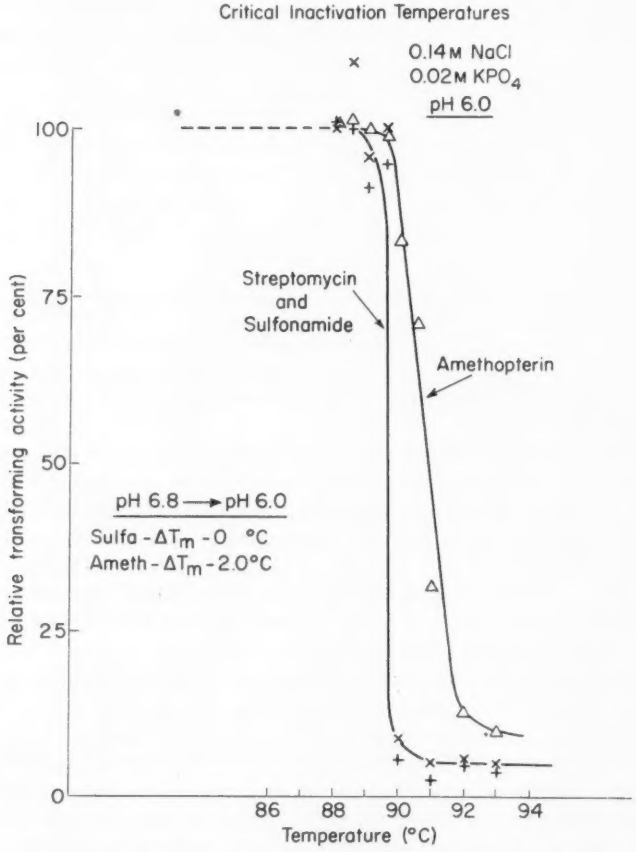


FIG. 6.—30 μg/ml DNA dissolved in buffered saline at pH 6.0. Solutions cooled in ice bath. Symbols and determination of relative transforming activity as described in Figure 1.

(Fig. 1) but not when buffering was at pH 6.8 (Figs. 4 and 5). A different demonstration of preferential pH sensitivity of the amethopterin marker will be presented in a later section.

Concomitant inactivation of a linkage group: In connection with interpretation of critical inactivation in terms of selective collapse of individual molecules, the behavior of three genetically linked markers is important to note. (Genetic linkage of two markers signifies that they are introduced together in what is apparently a single genetic event.) We have used here two subregions, Fa and Fd, of the complex sulfonamide resistance locus,[14] which are closely linked, that is, frequently transferred together from the double mutant Fad. The phenotype of the linked marker, Fad, is distinguishable from the less resistant phenotypes of the single markers, Fa and Fd. Streptomycin, S, is also linked to them, but "weakly"; that is, it is transferred with them but less frequently. The common interpretation of such frequency data is that Fa, Fd, and S are situated upon some common structure, with S "distant" from Fa and Fd, which are "closer" to each other.

It is seen in the data of Figures 1, 3, 4, and 5 that all three markers, Fd, Fad, and S are inactivated at the same T_c. The independent K and A markers have different T_c's, with a total 4° differential. It will be of interest to discover new markers linked to K and A and learn whether they are predictably inactivated at the respective T_c points of these latter. On the other hand, of 100 to 200 DNA molecules contained in a bacterial cell, it must be expected that similar but different molecules may have the same T_c. This might be the reason that canavanine resistance is inactivated at the same temperature as the F and S markers although it is not known to be genetically related to their linkage group.

Nature of the inactivated product: We have mentioned that inactivated DNA has always retained appreciable residual activity, constant within individual experiments. In all of the experiments presented until now, the heated samples of DNA were relatively quickly cooled, either in an ice bath or within 3–5 min in air at room temperature (usually about 25°C). The interesting description by Marmur and Lane[13] and Doty *et al.*[15] of reactivation and renaturation of heat-inactivated P-DNA upon very slow cooling led us to reexamine the effect of such variation in cooling rates as might have occurred in our studies. In the experiments presented in Figures 4 and 5, identically heated samples were therefore cooled respectively at 0° and at (summer) room temperature (over 30°C). There is a difference in the residual level to which the marker activities fall, this being over 25 per cent in the samples cooled relatively slowly—but still within a few minutes (Fig. 5). We may then be observing some reflection of the phenomenon seen in the renaturation experiments.

Marmur and Lane[13] propose that DNA while collapsing dissociates into two single strands (both biologically inactive), which with slow cooling reunite with complementary strands to reform an active double helical structure. Reactivation is in part considered to be reunion because it is favored by high ionic strength and by high concentration of DNA. According to this interpretation, material of reduced activity should be a mixture of intact (either undamaged or reconstituted) molecules and inactive separated strands.

The activity of our heated material, which has been relatively rapidly cooled, is as great as that obtained by Marmur and Lane for material reactivated through

slow cooling (usually 10 to 25 per cent of original activity). Nevertheless, closer examination of our partially active, heated material does not indicate that it has been formed by strand reunion. The residual activity level is not sensitive to ionic strength in the region between 0.015 *M* and 0.15 *M* NaCl. Neither are the residual activities influenced by increasing the temperature considerably above the T_c. Strand separation should be more efficient at higher temperatures, and for rapidly cooled DNA there should be a continual decline in activity at increasing temperatures, unless of course a fortuitous compensating effect occurs simultaneously.

More serious still, if residually active DNA were the product of strand separation and renaturation, it should behave like a mixture of intact and inactive DNA's. We have presented biological activities measured at cell-saturating concentrations of DNA (plateau activities) which are essentially independent of concentration. We did however study carefully the concentration-response curves for a number of inactivated samples. Whether measured just below, just at, or above the T_c point, relative activities were the same at plateau concentrations as in the lower concentration-dependent region. (Similar results were obtained for both inactivated and reactivated P-DNA by Marmur and Lane.[13]) A mixture of fully active intact molecules with totally inactive collapsed molecules would give specific activities at low concentration reflecting the number of active molecules. But when enough of the mixture is used to saturate the cells with competent DNA, the activity level would be determined by the ratio of intact marker/total competent DNA.[16] If the intact DNA in this mixture is fully active (either undamaged or renatured) and represents the same proportion of marked to unmarked molecules as does the untreated DNA, it should then have the same plateau activity as this latter. This is not the case for the residual activity which we observe (20-fold increase in heated-DNA concentration giving no higher activity), nor does it seem to be for the regained activity of heated DNA. Thus, active marker DNA and total competent DNA are not being reduced in proportion.

Neither is the competence of DNA to be bound to cell sites increased by heating so that it inhibits more effectively the transformation by the remaining active marker material. When critically heat-inactivated DNA is tested in admixture with intact DNA, it shows a loss of its competence. A P-DNA bearing sulfonamide marker was heated and used in competition with equal concentrations of intact streptomycin- and micrococcin-marked DNA (Fig. 7). The sulfonamide DNA inhibited transformation by the latter two markers 50 per cent in a 1:1 mixture before heating, but as it was heated, the inhibition decreased linearly within the same range of temperatures in which our group of known markers are inactivated. Thus, the competence of the total DNA present to react with cell sites falls rapidly and progressively in the critical temperature range.

A much more satisfactory model to explain our results would have *incomplete* separation of strands at and above the critical temperature, producing a readily renaturable molecule retaining a sufficient number of hydrogen-bonded points of contact to hold the two strands together in one or more regions. Such a molecule would be damaged only in its organization but would be chemically and possibly even genetically intact. The residual activities of such doubly stranded but collapsed molecules would reflect their lowered affinities for the bacterial cell with

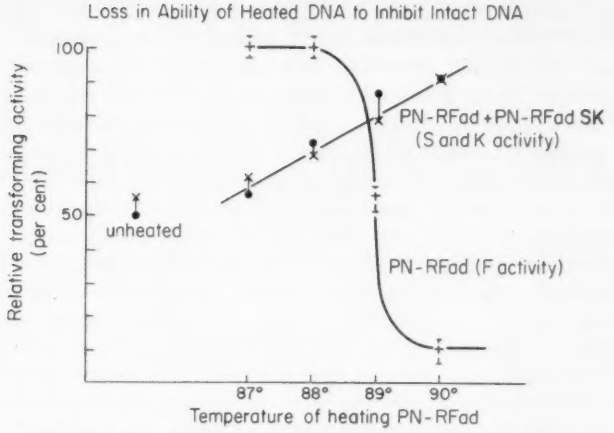


FIG. 7.—The Fad-marked P-DNA is heated at indicated temperatures to give the inactivation profile for sulfonamide (Fd) (+). A 1:1 mixture of *unheated* Fad-marked P-DNA with FadSK-marked P-DNA gives 50 per cent lowered plateau activity for both streptomycin (S) (×) and micrococcin (K) (●). *Heated* Fad-marked P-DNA mixed 1:1 with intact FadSK-marked P-DNA shows loss in inhibition as seen by increase in plateau activities of (S) (×) and (K) (●).

retention of much or all of their genetic potentialities. Such a mechanism in no way conflicts with the critical nature of the temperature transition. The concentration-response curve for the partially active, partially damaged molecules should reflect equal lowering of both specific and plateau activities, and such molecules would not be expected to compete successfully with fully intact DNA.

Doubly stranded molecules such as we have just pictured, still containing some regions of pairing, should have a high probability of renaturation compared with entirely separated single strands. Indeed, even if single strands are assumed to reunite, there must be partially anchored, two-stranded stages in their renaturation. Some of the factors tending to effect completion of the repairing process would be the same as those which tend to favor collision frequency in independent molecules. For it has often proven possible to analyze the behavior of linear high-molecular substances in terms of the behavior of a number of individual segments which, taken some distance apart, can be considered independent enough to be treated physically as separate molecules. Viewed in this way, partially attached DNA strands should be more likely to undergo complete repairing at higher ionic strengths, in part through the shielding effects of counter-ions on chain interactions. Most pertinent of all is the possibility that increased DNA concentration might provide an environment in which segments of the anchored complementary chains, having shorter mean free paths in the presence of repulsion by other DNA molecules, would tend to find each other more frequently and complete more fully their repair. Such effects could be expected at the same low DNA concentrations in which molecular interaction is detected by anomalies in sedimentation and viscosity effects. If such were the case, those stages of renaturation which are affected by high ionic strength or total DNA concentration may be intramolecular as well as intermolecular processes.

Subcritical Inactivation of P-DNA.—We have referred several times to an entirely different type of heat inactivation. During the early phases of this work, we suspected that certain apparent inconsistencies in the selective inactivation patterns obtained for the various markers, as well as in the temperatures required to produce inactivation, resulted from the occurrence of more than one kind of inactivating process. An exploratory study of the kinetics of inactivation at several temperatures at and below the critical point revealed that there were indeed two types of inactivation: the very rapid critical inactivation described above and a subcritical inactivation, which usually occurred more slowly and whose temperature dependence resembled that for a chemical reaction rather than a phase transition. The selective inactivation patterns for the two reactions were extremely different and the two reactions might be seen separately from one another, or superimposed, depending upon the intactness of the unheated DNA, the pH, and the temperature and time of heating. In particular, whether or not inactivation was seen below the critical temperature and how sharp the critical transition was depended upon the extent to which the subcritical type of inactivation was occurring.

Figure 8 shows the rate of inactivation of a P-DNA preparation a few degrees below the critical temperatures. As was the case when the activity-temperature profiles in Figures 1 and 3 were being determined, the P-DNA concentration is 50 μg/ml, the NaCl concentration is 0.15 M, and the solution is unbuffered but measured at pH 6.1 at room temperature. The temperature was maintained constant at 85°C in this experiment and the time scale is now in hours rather than minutes. Within the relatively short interval of 10 to 15 min, which is sufficient to produce complete inactivation at the higher critical temperatures, no detectable inactivation occurs. After a lag period of an hour's duration, the activities decline slowly. The contrast in the kinetics of this slow, subcritical inactivation and the very rapid critical inactivation becomes even more pronounced when we look at the relative inactivation rates for the different genetic markers. The Fd sulfonamide marker is the most resistant to inactivation. Streptomycin and micrococcin activities decay more rapidly and parallel each other. The activity of the complex pair of linked sulfonamide resistance markers (Fad) falls off most rapidly with time. Again, there is selective

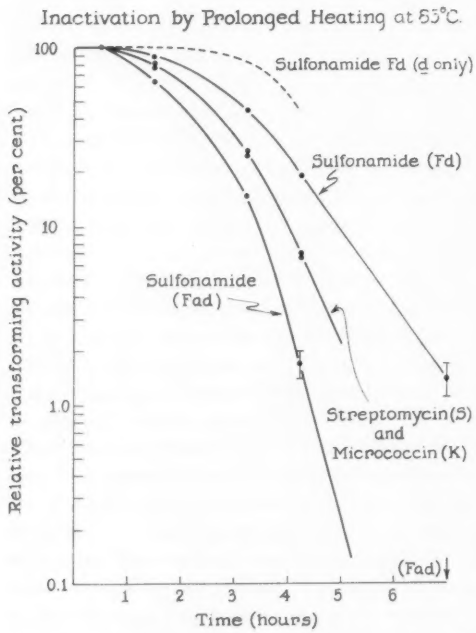


FIG. 8.—50 μg/ml P-DNA dissolved in 0.15 M NaCl heated for specified times at 85°C.

inactivation for one marker compared with the others; however, there is no particular correlation between the sensitivities of the markers in the fast critical inactivation and in the subcritical inactivation at lower temperatures. If we order the markers according to decreasing sensitivity to heat inactivation, we obtain the following patterns:

Critical Inactivation: $K > Fd{=}Fa{=}Fad{=}S{=}C > A$ (Increasing T_c →)

Subcritical Inactivation: $Fad > A > S{=}K{=}C > Fd{=}Fa$ (Decreasing Rate →)

Although both of these inactivation patterns are brought about by heating DNA solutions, the heating is obviously producing different types of damage in the two cases. The very nature of the exponential decay in activity below the critical temperatures (after a lag period) suggests that a cumulative type of damage is involved, such as the breaking of primary chemical bonds. There is no evidence at all here for a residual level: activity continues to fall below 0.1 per cent.

The most labile chemical bond in the DNA molecule under these conditions is the purine-deoxyriboside bond, which is known to be acid-sensitive. Although the conditions employed are very mild compared with those used for the efficient removal of purines, if full activity requires intactness of the entire sequence of bases of a genetic locus, the actual loss of purines which would give a measurable decrease in biological activity would be very small indeed. Were such the case, the small percentage of purines that need be randomly removed to produce almost complete inactivation would be difficult to detect chemically and we have not attempted such an analysis. It has been shown that prolonged heating of neutral solutions of calf thymus DNA does produce detectable quantities of free purines and recently data have been made available to us which give evidence for the release of purines from DNA heated for shorter periods.[17]

The effect of moderate changes in pH on the inactivation rates lends further support to the depurination reaction and is seen in Figure 9. Each curve consists of only two or three points but the data are sufficient to convey the trend of decreased rate in going from pH 5.4 to 7.8. The differences observed in the inactivation rates at pH 6.0 and 5.4 are particularly striking, the multiple-hit nature of the curves being no longer apparent at pH 5.4. Were a critical inactivation attempted under these conditions, more than 50 per cent activity could be lost by depurination alone.

The existing data on DNA indicate that both the adenine and the guanine deoxyriboside bonds are hydrolyzed at the same rate, so there is no basis for explaining selective inactivation in this way. The random splitting of a small percentage of purines does, however, provide a satisfactory mechanism for selection merely on the basis of the relative sizes of the markers.

The complex sulfonamide marker, Fad, can be considered to occupy a relatively large genetic region, encompassing as it does both the Fa and the Fd markers. In a normal transformation experiment with intact DNA, 50 per cent of the total transformants to Fd also are transformed to Fa, and the two linked markers are

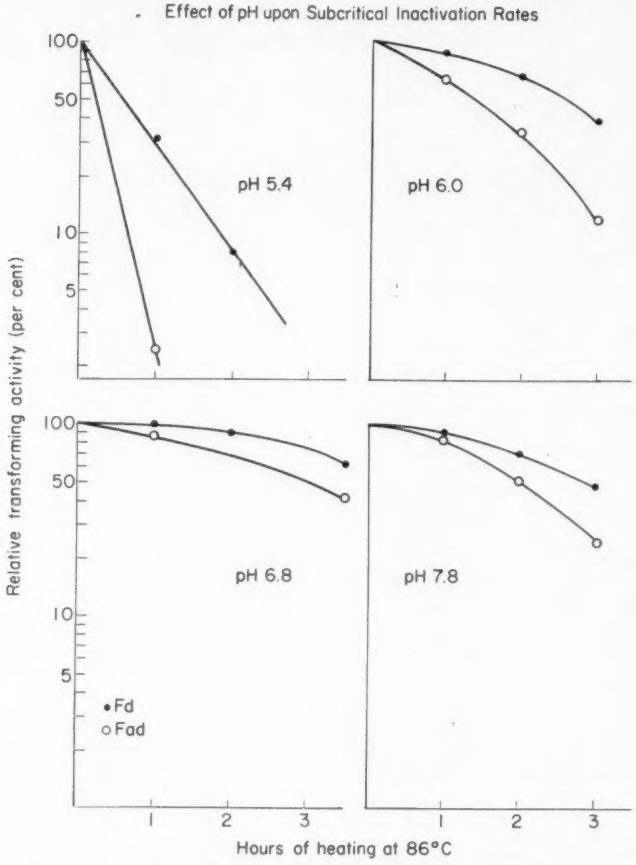


FIG. 9.—30 μg/ml P-DNA dissolved in saline-phosphate at the specified pH values. Solutions heated at constant temperature, 86°C, for the specified periods of time.

therefore considered to reside relatively close to each other in terms of genetic map distances. The marker most sensitive with respect to subcritical heat inactivation is the large complex Fad, and the most resistant ones are its components, Fa and Fd. When the Fd marker retains 10 per cent of its initial activity, the linked pair Fad is inactivated tenfold more. In contrast, the critical inactivation of Fd and Fad, as well as the more weakly linked S marker, occurred at the same temperature, and Fa and Fad were always inactivated to the same extent. The linkage between Fa and Fd in collapsed material at the 10 per cent residual activity level is the same as that for intact DNA.

When linkage of two traits is seen in transformation experiments, it has seemed reasonable to assume that these two traits are associated with a single particle or molecule of DNA. Nevertheless, in this system, as in viral and microbiological genetics generally, the material or molecular basis for linkage remains essentially

an assumption derived from *frequencies* of coincidence. What we believe we are demonstrating here is that the rearrangement occurring at the critical temperature for a particular molecule inactivates the entire molecule and with it an entire linkage group. On the other hand, subcritical heat inactivation, or depurination, inactivates small regions within the DNA molecules, even within a closely linked pair of markers, and its selectivity depends upon the distribution of these regions.

The quantitative behavior of the three sulfonamide markers, Fa, Fd, and Fad, with subcritical heating provides additional information. Throughout these experiments, quantitative measurements of Fa transformants have been limited but are adequate to show that Fa and Fd are inactivated at similar rates. In Table 1 are given some complete data for the three sulfonamide transformant

TABLE 1

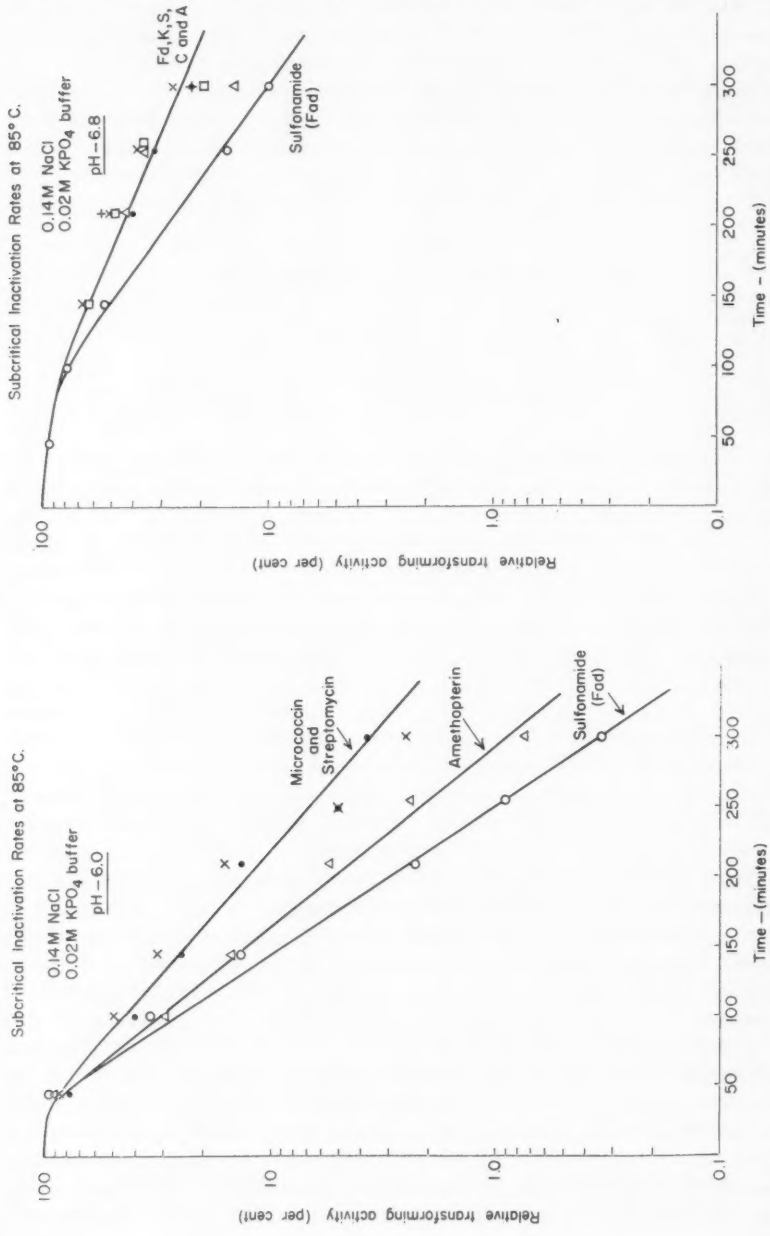
RELATIVE SURVIVAL OF LINKED MARKERS AT 85°C

Conditions of heating	Total Fa	Total Fd	Observed Fad	Product Fa × Fd
pH 5.4—1 hr	0.16	0.25	0.03	0.04
pH 6.1—3 hr	0.40	0.41	0.14	0.16
pH 6.8—3 hr	0.81	0.75	0.65	0.61
pH 7.8—2 hr	0.58	0.66	0.52	0.39
pH 7.8—3 hr	0.60	0.47	0.26	0.28

types. The fourth column gives the calculated product of surviving activity for transformants to Fa and Fd and is to be compared with the third column which gives the measured surviving Fad activity. The good agreement between the two is what is expected if the inactivating damage is occurring randomly at regions along a DNA chain small in relation to the size of a genetic locus. Furthermore, such agreement with the predicted random probability of damaging either the *a* or *d* region of the Fad locus should only be seen if the *a* and *d* regions are essentially adjoining each other, as perhaps they are. A further consequence of these results is that a damaged DNA molecule which has lost ability to transfer one of its genetic regions, e.g., Fa, is still capable of transferring its remaining undamaged regions, e.g., Fd. This would follow if the inactivation of any two regions within a molecule occur independently of each other and if a molecule partially damaged in this way may be incorporated by the bacterial cell. The work of Lerman and Tolmach[8] with P^{32}-labeled P-DNA lends support to this hypothesis. At about the same time, a similar analysis of the relative sizes of a series of eight linked amylomaltase markers was carried out by Lacks and Hotchkiss[18] with good qualitative correlation between the genetic and heat inactivation data.

Figures 10 and 11 give more complete inactivation curves comparing pH 6.0 with 6.8. The heated solutions were buffered in these experiments, and the amethopterin and canavanine markers, in addition to those included in Figure 8, were measured. The exponential nature of the decay in activities after the lag period is more clearly seen in these curves than could be deduced from Figure 8. An interesting observation is the abnormal increase in inactivation rate of the amethopterin marker at pH 6.0 as compared with pH 6.8. Streptomycin, micrococcin, and sulfonamide, Fad, all show a smaller and uniformly increased rate of inactivation at the lower pH. At pH 6.0 amethopterin becomes almost as sensitive as the Fad marker. The pronounced pH dependence of the amethopterin marker has already been seen in the critical inactivation data. We can only

speculate that the two observations may have arisen from a common structural basis. It may be that a high temperature dependence for depurination of certain base distributions, such as that present in the amethopterin marker region, can



FIGS. 10 (*left*) and 11 (*right*).—30 μg/ml P-DNA dissolved in buffered saline at pH 6.0 or pH 6.8. Temperature maintained constant at 85°C. for specified periods of time. Symbols and determination of relative transforming activity as described in Fig. 1.

at low pH bring the depurination reaction into the time and temperature region of the critical transition.

Summary.—Pneumococcal transforming DNA when heat inactivated shows two very different selective inactivation patterns for five drug-resistance markers, reflecting two different inactivation processes. When it is heated briefly at successively higher temperatures, a critical inactivation occurs, different genetic markers being inactivated at different characteristic critical temperatures. Heating for extended periods of time at subcritical temperatures results in a slow inactivation (presumably depurination) with different genetic markers being inactivated at different rates. When correlation is made with the physical changes as well as with the behavior of linked and unlinked groups of genetic markers, the conclusion is drawn that the critical inactivation process involves a physical collapse of entire molecules and linkage groups, while the subcritical inactivation involves the damage of small regions within molecules, selectivity depending upon their relative size. The kinetics of the two types of inactivation allow separation of the processes by the proper choice of the time and temperature of heating. The structural map of the DNA markers chemically and physically indicated in this way is consistent with the known genetic map of these markers.

We wish to acknowledge discussions of this work throughout the past several years with Drs. Paul Doty and Julius Marmur and some months ago with Dr. William Ginoza. We are particularly indebted to Dr. Ginoza and to Dr. Bruno H. Zimm, who, knowing of our early involvement in these studies, kindly arranged simultaneous publication in these PROCEEDINGS (see pp. 633–652).

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*DEOXYRIBONUCLEIC ACID-DEPENDENT INCORPORATION OF NUCLEOTIDES FROM NUCLEOSIDE TRIPHOSPHATES INTO RIBONUCLEIC ACID**

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After the discovery of polynucleotide phosphorylase[1] a search was started in several laboratories for other enzymes which might be concerned with RNA[2] synthesis. The main reason for this was that, contrary to DNA polymerase,[3] polynucleotide phosphorylase did not appear to provide a mechanism for nucleic acid replication. Furthermore, since RNA is supposedly the carrier of genetic information from DNA to protein, a system for RNA replication might be expected to require DNA for activity. As a result of this search animal and bacterial cells (rat liver nuclei,[4] *Micrococcus lysodeikticus*,[5] *Escherichia coli*[6, 7]) were found to contain enzymes which bring about a DNA-dependent incorporation of nucleotides from ribonucleoside triphosphates into RNA. The need for DNA and the fact that all four triphosphates (ATP, GTP, UTP, CTP) were required for maximal incorporation suggested a replication mechanism. However, while participation of polynucleotide phosphorylase in the observed incorporations was unlikely, it was not rigorously excluded, nor was the possibility ruled out that DNA might act indirectly by inhibiting nucleases present in the enzyme preparations.

This paper reports on preliminary studies with enzyme preparations from two microorganisms, *Lactobacillus arabinosus* and *Azotobacter vinelandii* which, in agreement with the above observations, were found to catalyze a DNA-dependent incorporation of labeled ATP or GTP into RNA from mixtures of ATP, GTP, UTP, and CTP. Participation of polynucleotide phosphorylase was ruled out by (*a*) absence of this enzyme from *L. arabinosus* extracts, confirming earlier observations,[8] and (*b*) inorganic phosphate inhibition of its RNA-synthesizing activity in *A. vinelandii* preparations without affecting nucleotide incorporation from nucleoside triphosphates. Indications were also obtained that DNA is directly involved in the latter reaction.

Materials.—Enzyme preparations: *L. arabinosus* (ATCC *L. plantarum*, No. 8014) and *A. vinelandii* (ATCC, No. 9104), obtained from the American Type Culture Collection, Washington, D. C., were grown essentially as described by Stumpf and Horecker[9] and Newton *et al.*,[10] respectively. If not used immediately, the cells were stored at −18°. Extracts were prepared by sonic disintegration of cell suspensions (2.5–3.0 g in 10 ml of 0.1 *M* Tris–HCl buffer, pH 7.0) in a 10 *KC* Raytheon oscillator for 10 min at 2° to 5°, centrifugation at 25,000 × *g* for 15 min in the cold room, and dialysis (3–5 hrs at 4°) of the clear supernatant fluid against 4 liters of 0.01 *M* Tris–HCl buffer, pH 7.0. The protein content of the extracts, determined by the biuret method[11] was 20–30 mg/ml. *A. vinelandii* extracts were occasionally prepared by disrupting the cells with glass beads in the Waring blendor as described by La Manna and Mallette.[12] Ammonium sulfate fractionation of the extracts was carried out at pH 7.4 essentially as described by Grunberg-Manago *et al.*[13] The fractions were dissolved in a small volume of 0.01 *M* potassium phosphate buffer, pH 7.4, and dialyzed with stirring overnight at 0° against 0.033 *M* succinate buffer, pH 6.3, containing 0.5×10^{-3} *M* cysteine.

The polynucleotide phosphorylase fractions used for some experiments were prepared from *A. vinelandii* as outlined by Ochoa *et al.*[14]; the purification procedure will be described in detail elsewhere. The specific activity of these fractions (μmoles/mg protein/15 min at 30°) as assayed by

P^{32}-ADP exchange[13] had decreased on prolonged keeping. They did not catalyze nucleotide incorporation from nucleoside triphosphates whether in the absence or presence of DNA.

Purine nucleotide pyrophosphorylase, nucleoside monophosphokinase, and pyruvic kinase were used for the preparation of C^{14}-labeled GDP and GTP. An acetone powder extract of pig liver, prepared as described by Way and Parks,[15] was used as the source of the purine nucleotide pyrophosphorylase. The dialyzed extract was cooled to 0° and adjusted to pH 5.0 by the gradual addition of 1.0 *M* KH_2PO_4; the precipitate was collected by centrifugation in the cold, taken up in 0.01 *M* Tris–HCl buffer, pH 8.0 and insoluble material was removed by centrifugation.[16] Nucleoside diphosphokinase was prepared from Brewer's yeast by the method of Lieberman *et al.*[17] through the ethanol fractionation step. The solution was then adjusted to pH 1.5 with 1.0 *N* HCl, heated in a boiling water bath for 4 min, and cooled after adjusting the pH to 6.8 with 1.0 *N* KOH. The heavy precipitate was removed by centrifugation and the supernatant brought to 90 per cent saturation with solid ammonium sulfate. The precipitate was collected by centrifugation and dissolved in a small volume of 0.025 *M* glycylglycine buffer pH 7.4. Pyruvic kinase (crystalline) and adenylic kinase (myokinase) were purchased from C. F. Boehringer and Sons, Mannheim, Germany. The latter enzyme was used for the preparation of P^{32}-labeled ATP.

Crystalline pancreatic RNAase and DNAase were obtained from the Worthington Biochemical Corporation, Freehold, New Jersey; *Crotalus adamanteus* venom from Ross Allen's Reptile Institute, Silver Springs, Florida.

Labeled nucleotides: GMP-8-C^{14} was prepared enzymatically from guanine-8-C^{14} (New England Nuclear Corporation, Boston) and PRPP (Pabst Laboratories, Milwaukee) with adenine nucleotide pyrophosphorylase. The reaction mixture containing (in millimoles) Tris–HCl buffer, pH 9.0, 6; $MgCl_2$, 1.2; reduced glutathione, 1; guanine-8-C^{14}, 0.02; PRPP, 0.06, and pig liver enzyme with 100 mg of protein in a final volume of 169 ml, was incubated for 40 min at 37°. After heating at 100° for 3 min, the precipitate was removed by centrifugation and the supernatant passed through a Dowex-1 (formate) column (25 × 1.2 cm). GMP-8-C^{14} was isolated by gradient elution according to Hurlbert *et al.*[18] lyophilized and dissolved in water. Yield about 80 per cent. The solution was adjusted to pH 6.0.

For preparation of GDP-8-C^{14} and GTP-8-C^{14} a reaction mixture containing (in μmoles) phosphate buffer, pH 7.4, 500; $MgCl_2$, 20; ATP, 20; GMP-8-C^{14}, 10, and yeast nucleoside monophosphokinase with 6 mg of protein in a final volume of 10 ml, was incubated for 30 min at 37°. The reaction was stopped by addition of 1 ml of ice-cold 1.5 *N* perchloric acid and the pH adjusted to 6.0 with 1.0 *N* KOH after cooling the mixture to 0°. The precipitated potassium perchlorate was removed by centrifugation and the nucleotides isolated by column chromatography (Dowex-1, formate) as above. The enzyme preparation contains nucleoside diphosphokinases and the procedure yielded C^{14}-labeled GDP (3 μmoles) and GTP (2 μmoles) along with unchanged GMP (1.5 μmoles). The labeled GDP and GTP were adsorbed on charcoal, eluted with ethanol-ammonia-water (50:3:47), lyophilized, and dissolved in water. The specific radioactivity of each nucleotide was in the neighborhood of 1.5×10^6 cpm/μmole. When only GTP-C^{14} was required, the incubation was carried out in the additional presence of pyruvic kinase and phosphoenolpyruvate. In a typical preparation a reaction mixture containing (in μmoles) phosphate buffer, pH 7.4, 250; $MgCl_2$, 90; ATP, 15; GMP-8-C^{14}, 10; phosphoenolpyruvate, 30; yeast nucleoside monophosphokinase with 4.6 mg of protein, and 1.2 mg. of crystalline pyruvic kinase in a final volume of 20 ml (incubation, 40 min at 37°), yielded about 8 μmoles of GTP-8-C^{14}, specific radioactivity 1.5×10^6 cpm/μmole.

ATP labeled with P^{32} in the α-phosphate (adenosine-P^{32}-P-P) was prepared enzymatically from AMP^{32}, ATP, and phosphoenolpyruvate, in the presence of adenylic kinase and pyruvic kinase. AMP^{32} was synthesized by the method of Tener[19] from P^{32}-labeled cyanoethylphosphate and isopropylideneadenosine (Aldrich Chemical Company, Milwaukee) and isolated by ion-exchange chromatography (Dowex-1 formate), according to Bergvist and Deutsch.[20] We are indebted to Dr. R. W. Chambers for help with this preparation. A reaction mixture containing (in μmoles) phosphate buffer, pH 7.4, 250; $MgCl_2$, 350; AMP^{32} (5×10^7 cpm/μmole), 27; ATP, 5; phosphoenolpyruvate, 54; adenylic kinase, 1.75 mg; and pyruvic kinase, 3.5 mg, in a final volume of 14 ml, was incubated for 30 min at 37°. After cooling in ice, the nucleotides were isolated by ion-exchange chromatography[18] as described above for GDP-C^{14} and GTP-C^{14}. 15 μmoles of P^{32}-labeled ATP (3×10^7 cpm/μmole) were obtained.

Other preparations: Bacterial DNA was prepared by a new method to be described in detail elsewhere. It is based on the observation that ammonium sulfate supernatant fractions of bacterial extracts at 70-per cent saturation contain up to 85 per cent of the DNA of the extract with only traces of RNA and protein. DNA can be isolated from such supernatants in a high degree of polymerization and purity after removing the ammonium sulfate by exhaustive dialysis against running deionized water. We are indebted to Dr. E. Chargaff, Columbia University, and Dr. S. S. Cohen, University of Pennsylvania, for gifts of spleen and T_4 bacteriophage DNA, respectively. *A. vinelandii* RNA was isolated from dialyzed ammonium sulfate fractions (33–46% saturation), which contain little DNA, after removal of the protein with isoamylalcohol-chloroform. We are indebted to Dr. F. W. Allen, University of California Medical Center, San Francisco, and Dr. K. K. Reddi of this department for gifts of yeast RNA[21] and tobacco mosaic virus (TMV) RNA, respectively. ADP-8-C^{14} and ATP-8-C^{14} were obtained from the Schwarz Laboratories, Mt. Vernon, N. Y.; all other nucleotides and PRPP from the Pabst Laboratories, Milwaukee.

Methods.—Enzyme assays: In the standard assay for incorporation of labeled nucleotides into RNA each sample, in a final volume of 0.25 ml contained (in μmoles) Tris–HCl buffer, pH 8.0, 20; $MgCl_2$, 5; inorganic phosphate, 1.5; ATP-8-C^{14} (specific radioactivity about 5×10^6 cpm/μmole), 0.1; nonlabeled GTP, UTP, and CTP, each 0.5, and enzyme. Incubation, 10 min at 37°. Deviations from these conditions are noted in the table and figure legends. After incubation, the samples were deproteinized with 1 ml of 5 per cent trichloroacetic acid and worked up as described by Weiss.[4] Occasionally GTP-8-C^{14} (specific radioactivity 4×10^4 to 5×10^5 cpm/μmole) was substituted for ATP-8-C^{14}, and nonlabeled ATP for GTP. To assay for the incorporation of nucleotides due to polynucleotide phosphorylase, inorganic phosphate was omitted and ribonucleoside 5′-diphosphates were substituted for the corresponding triphosphates. In this case, however, all diphosphates were present in equimolar (each 0.5×10^{-3} *M*) concentrations. Under the conditions of these assays the amount of labeled RNA increased almost linearly with time for 10–15 min and decreased thereafter, indicating degradation by nucleases present in the enzyme preparations. However, nucleases do not interfere with the P^{32}-ADP exchange assay[13] for polynucleotide phosphorylase. The exchange assay was therefore used when knowledge of the true polynucleotide phosphorylase activity of the preparations was required.

Determination of RNA and DNA: 0.5 ml of extract or enzyme fraction was treated with 2.5 ml of 0.6 *N* $HClO_4$; the mixture was stirred in an ice bath for 10 min and centrifuged at $25{,}000 \times g$ in the Servall angle centrifuge. The precipitate was washed once with 2.5 ml of 0.6 *N* $HClO_4$, once with 5 ml of ethanol and once with 5 ml of ethanol-ether (3:1). For the determination of RNA the perchloric acid precipitate was dissolved in 2 ml. of 1.0 *N* KOH and the solution incubated for 18 hrs at 37°. The DNA was then removed by precipitation with 3 ml of 5 per cent trichloroacetic acid after adding 0.05 ml of crystalline serum albumin (50 mg/ml) as carrier and 0.4 ml of 6.0 *N* HCl, standing for 5 min at 0°, and centrifugation. Pentose was determined in the supernatant by the orcinol reaction.[22] For the determination of DNA, the RNA was first hydrolyzed with RNAase. The washed perchloric acid precipitate was dissolved in 3 ml of 0.1 *M* ammonium acetate (pH 8.0) and, after adding 0.1 ml of 0.1 *M* EDTA (to inhibit any DNAase that might be present) and 0.1 ml of crystalline pancreatic RNAase (0.5 mg/ml), the mixture was incubated for 2 hrs at 37° with occasional stirring. The DNA was then precipitated by addition of 3 ml of ice-cold 5-per cent trichloroacetic acid and the precipitate collected by centrifugation after standing for 15 min at 0°. Upon washing with 5 ml of 5 per cent trichloroacetic acid, and heating for 15 min at 90° with 2 ml of the same solution, the mixture was centrifuged and deoxypentose determined in the supernatant by the diphenylamine reaction according to Burton.[23] Authentic samples of RNA and DNA, put through the same treatment as the experimental samples, were used as standards.

Isolation and hydrolysis of labeled RNA: C^{14}- or P^{32}-labeled RNA was isolated from incubation mixtures by Kirby's method[24] and dialyzed exhaustively against 0.05 *M* KCl. Alkaline hydrolysis was carried out in 0.3 *N* KOH for 18 hrs at 37°. The mixture was neutralized with perchloric acid and the precipitate of potassium perchlorate removed by centrifugation. DNAase hydrolysis was carried out in samples (final volume, 1.0 ml) containing labeled RNA; Tris–HCl buffer, pH 7.0, 20 μmoles; $MgCl_2$, 6 μmoles, and enzyme, 120 μg. Conditions for RNAase hydrolysis were the same except for the omission of $MgCl_2$.

Separation of labeled nucleotides: P^{32}-labeled nucleotides from alkaline hydrolysis of P^{32}-labeled

RNA were isolated by ion-exchange chromatography on Dowex-1 (formate) columns as in previous work.[25] For the separation of AMP-8-C^{14} and adenosine-8-C^{14} released by alkaline hydrolysis of C^{14}-labeled RNA, aliquots of the neutralized hydrolysate were subjected to electrophoresis on Whatman No. 3 *MM* filter paper strips (56 × 9 cm) in 0.02 *M* citrate buffer, pH 3.5, for 5 hrs at 1000 volts. For radioactivity measurements the adenosine and AMP spots were eluted with water.

Results.—Incorporation of nucleotides from nucleoside triphosphates into RNA: In extracts of tissues or bacteria, nucleoside triphosphates are partially converted to the corresponding diphosphates. This occurs through formation of ADP and AMP from ATP, by ATPase and adenylic kinase, and transfer of phosphate from nucleoside triphosphates to AMP and ADP catalyzed by nucleoside mono- and diphosphokinases. Since polynucleotide phosphorylase catalyzes synthesis of RNA from nucleoside diphosphates, incorporation of labeled nucleotides from nucleoside triphosphates into RNA by crude enzyme preparations could be due, at least in part, to this enzyme. For the present work it was therefore essential to rule out polynucleotide phosphorylase at the outset.

Extracts of *L. arabinosus* have previously been found to be essentially free of polynucleotide phosphorylase;[8] this observation was confirmed by P^{32}-ADP exchange and poly A phosphorolysis assays at various pH values. The possibility of assaying for RNA-synthesizing enzymes other than polynucleotide phosphorylase when this enzyme is present was next investigated. As shown in Figure 1 the incorporation of labeled ADP-8-C^{14} into acid-insoluble polynucleotides with purified polynucleotide phosphorylase was inhibited by inorganic phosphate. With ADP-C^{14} alone complete inhibition occurred at 0.4×10^{-3} *M* phosphate (curve 1). With ADP-C^{14} and non-labeled GDP, UDP, and CDP in equimolar concentrations, phosphate increased slightly the incorporation up to 0.2×10^{-3} *M* and inhibited completely at 10^{-3} *M* (curve 2). The smaller incorporation from the mixture of nucleoside diphosphates is due to the previously observed[13] inhibition of polynucleotide synthesis by GDP. The occurrence of incorporation in the latter case at phosphate concentrations which completely repress incorporation from ADP-C^{14} alone is undoubtedly related to the fact that polynucleotide copolymers are more resistant than homopolymers to phosphorolysis[13, 26] As expected from the data in Figure 1, incorporation of ADP-C^{14} into RNA with extracts and ammonium sulfate fractions of *A. vinelandii*, which are rich in polynucleotide phosphorylase, was inhibited by inorganic phosphate (Table 1). Under these conditions, there was incorporation of ATP-C^{14} in the presence of nonlabeled GTP, UTP, and CTP (Table 1, exp. 2). With both *L. arabinosus* and *A. vinelandii* extracts incorporation of ATP-C^{14} or GTP-C^{14} was maximal when the other three nucleoside triphosphates were present (Table 2).

Effect of DNA: On fractionation of *A. vinelandii* extracts with ammonium sulfate there was a loss of total activity although some activity was present in the 33–46% saturation fraction (middle fraction). The enzymatic specific activity of this fraction (Table 3, column 6) was only slightly higher than that of the initial extract. It was observed (Table 3, columns 3 and 4) that while the RNA content of the middle fraction was high, its DNA content was very low. In fact, up to 85 per cent of the extract's DNA was present in the supernatant at 70 per cent ammonium sulfate saturation. Addition of DNA markedly increased the activity of the middle fraction (Table 3, column 5). Figure 2 shows the time course of in-

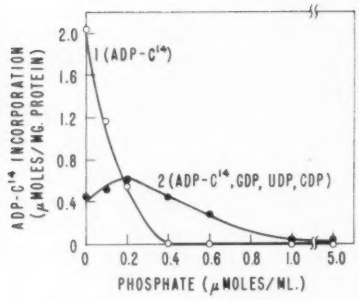

FIG. 1—Effect of inorganic phosphate on polynucleotide synthesis by polynucleotide phosphorylase from ADP-C^{14} alone (curve 1) or together with non-labeled GDP, UDP, and CDP (curve 2). Samples contained (in μmoles) Tris buffer, pH 8.0, 20; $MgCl_2$, 1; ADP-C^{14} (10^5 cpm/μmole), 0.1, with or without GDP, UDP, and CDP, each 0.1; the indicated concentrations of orthophosphate; and purified *A. vinelandii* polynucleotide phosphorylase (gel eluate fraction, specific activity 25) with 4 μg of protein. Final volume, 0.2 ml; incubation, 10 min at 37°.

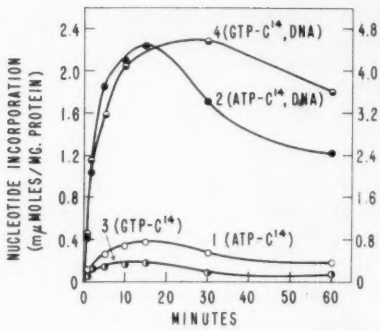

FIG. 2.—Time course of nucleotide incorporation from ATP-C^{14} or GTP-C^{14} into RNA in the absence and presence of DNA. Samples contained (in μmoles/ml) Tris buffer, pH. 8.5, 80; $MgCl_2$, 20; orthophosphate, 6; either ATP-C^{14} (5×10^4 cpm/μmole), 0.4, and non-labeled GTP, UTP, and CTP, each 2, or GTP-C^{14} (4×10^5 cpm/μmole), 0.4, and non-labeled ATP, UTP, and CTP, each 2; and 33–46 ammonium sulfate fraction of *A. vinelandii* with 10.6 mg of protein. *A. vinelandii* DNA (when present), 224 μg. Incubation at 37°. 0.25-ml aliquots were pipetted into 1.0 ml of 5% trichloroacetic acid at various time intervals. Curves 1 and 2, ATP-C^{14} without and with DNA, respectively. Curves 3 and 4, GTP-C^{14} without and with DNA, respectively. Ordinate, left, ATP-C^{14} experiment; right, GTP-C^{14} experiment.

TABLE I

EFFECT OF PHOSPHATE ON INCORPORATION OF NUCLEOTIDES FROM NUCLEOSIDE DIPHOSPHATES INTO RNA IN *A. vinelandii* EXTRACTS

Experiment no.	Enzyme, mg protein	Labeled nucleotide	Other nucleotides	Orthophosphate, μmoles	Nucleotide incorporation, mμmoles/mg protein
1	Extract, 1.0	ADP-C^{14}	None	0	0.43
		ADP-C^{14}	None	1.0	0.05
		ADP-C^{14}	GDP, UDP, CDP	0	0.42
		ADP-C^{14}	GDP, UDP, CDP	1.0	0.05
2	Extract, 2.4	ADP-C^{14}	None	1.5	0.02
		ADP-C^{14}	GDP, UDP, CDP	1.5	0.05
		ATP-C^{14}	None	1.5	0.06
		ATP-C^{14}	GTP, UTP, CTP	1.5	0.28
3	33–46 $(NH_4)_2SO_4$ fraction, 2.5	ADP-C^{14}	GDP, UDP, CDP	0	0.72
		ADP-C^{14}	GDP, UDP, CDP	1.5	0.08

Samples contained (in μmoles) Tris buffer, pH 8.0, 20; $MgCl_2$, experiment 1, 1; others, 5; ADP-C^{14} (5×10^5 cpm/μmole), 0.1 and, when present, nonlabeled GDP, UDP, and CDP, each 0.1 in experiments 1 and 2 and 0.5 in experiment 3, or ATP-C^{14} (5×10^5 cpm/μmole) 0.1 and, when present, nonlabeled GTP, UTP, and CTP, each 0.5. Enzyme and orthophosphate as indicated. Final volume, experiment 1, 0.2 ml; others, 0.25 ml. Incubation, 10 min at 37°.

corporation of ATP-C^{14} and GTP-C^{14} without and with added DNA. It may appear surprising that the incorporation of GTP-C^{14} was twice as high as that of ATP-C^{14}. This may be due to the fact that dephosphorylation of ATP by ATPase in the *A. vinelandii* ammonium sulfate fraction decreased the concentration of this substrate. The incorporation of ATP-C^{14}, as catalyzed by the middle am-

TABLE 2

DEPENDENCE ON OTHER NUCLEOSIDE TRIPHOSPHATES OF NUCLEOTIDE INCORPORATION FROM ATP-C^{14} OR GTP-C^{14} INTO RNA IN BACTERIAL EXTRACTS

Experiment no.	Extract, mg protein	Orthophosphate, μmoles	Labeled compound, μmoles	Conditions	Nucleotide incorporation, mμmoles/mg protein
1	*L. arabinosus*, 2.5	0	ATP-C^{14} (0.1)	Complete	0.08
				No GTP	0.02
				No UTP	0.04
				No CTP	0.02
				No GTP, no UTP, no CTP	0.01
2	*L. arabinosus*, 1.9	0	GTP-C^{14} (0.035)	Complete	0.20
				No ATP	0.02
				No UTP	0.03
				No CTP	0.07
3	*A. vinelandii*, 2.4	1.25	GTP-C^{14} (0.045)	Complete	0.52
				No ATP	0.32
				No UTP	0.20
				No CTP	0.19
				No ATP, no UTP, no CTP	0.01
4	*A. vinelandii*, 2.3	1.5	ATP-C^{14} (0.1)	Complete	0.60
				No GTP	0.12
				No UTP	0.17
				No CTP	0.18
				No GTP, no UTP, no CTP	0.08

Samples contained (in μmoles) Tris buffer, pH 8.0, 20; $MgCl_2$, 5; nonlabeled nucleoside triphosphates, each 0.5. Other additions as indicated. Final volume, 0.25 ml. Incubation at 37°, experiments 1 and 2, 15 min, experiments 3 and 4, 10 min. Specific radioactivity of ATP-C^{14}, 6×10^5 cpm/μmole; of GTP-C^{14}, experiment 2, 1.1×10^5, experiment 3, 5.3×10^5 cpm/μmole.

TABLE 3

RNA AND DNA CONTENT AND NUCLEOTIDE INCORPORATION FROM NUCLEOSIDE TRIPHOSPHATES INTO RNA WITH AMMONIUM SULFATE FRACTIONS OF *A. vinelandii*

Fraction	Protein, mg/ml	RNA, μg/mg protein	DNA, μg/mg protein	Nucleotide incorporation,* mμmoles/mg protein	
				Without DNA	With DNA
Extract	21.0	190	22.4	0.27	0.55
$(NH_4)_2SO_4$ 0–33	35.6	135	1.4	0.04	0.27
$(NH_4)_2SO_4$ 33–46	53.0	340	1.2	0.34	2.05
$(NH_4)_2SO_4$ 46–70	13.0	446	24.6	0.03	0.04

* Standard assay with the following amounts of protein: Extract, 2.1 mg; ammonium sulfate fractions, 0–33 2.2 mg; 33–46, 2.1 mg; 46–70, 2.0 mg; *A. vinelandii* DNA (when present), 56 μg.

monium sulfate fraction of *A. vinelandii*, was stimulated by DNA preparations of various origins while RNA had little or no effect (Table 4). These results do not exclude a possible effect of the latter since, as previously pointed out, the ammonium sulfate fraction contained large amounts of RNA. Other preparations of DNA (*L. arabinosus*, calf thymus, salmon sperm) were also active. Increasing the amount of DNA eventually led to inhibition of ATP-C^{14} incorporation but the minimal inhibitory concentration varied widely with different preparations.

As stated in the introduction it was possible that DNA might act indirectly by inhibiting nucleases in the enzyme preparations. This was ruled out in experiments with purified polynucleotide phosphorylase and ammonium sulfate fractions of *A. vinelandii*. In Figure 3A the time course of synthesis of RNA by polynucleotide phosphorylase from an equimolar mixture of ADP-C^{14}, GDP, UDP, and CDP was followed by measuring the incorporation of radioactivity into an acid-insoluble product. It may be seen that DNA had little or no effect on the

TABLE 4

EFFECT OF VARIOUS NUCLEIC ACIDS ON NUCLEOTIDE INCORPORATION FROM NUCLEOSIDE TRIPHOSPHATES INTO RNA

Nucleic acid addition, μg	Nucleotide incorporation,* mμmoles/mg protein	Change
None	0.28	...
TMV RNA, 34	0.21	−0.07
TMV RNA, 68	0.21	−0.07
Yeast RNA, 48	0.49	+0.21
Yeast RNA, 96	0.36	+0.08
A. vinelandii RNA, 112	0.49	+0.21
Spleen DNA, 42	0.69	+0.41
Spleen DNA, 84	1.31	+1.03
A. vinelandii DNA, 28	0.95	+0.67
A. vinelandii DNA, 56	1.72	+1.44
T_4 phage DNA, 34	1.17	+0.89
T_4 phage DNA, 68	0.93	+0.65

* Standard assay. Enzyme, 33–46 ammonium sulfate fraction of *A. vinelandii* with 2.65 mg of protein. Other additions as indicated.

inhibition caused by pancreatic ribonuclease. In Figure 3*B*, the time course of synthesis of poly AU from equimolar concentrations of ADP-8-C^{14} and UDP was followed in the same way. The enzyme preparations (purified polynucleotide phosphorylase and 33–46 ammonium sulfate fraction of *A. vinelandi*) were used in amounts such that the polynucleotide phosphorylase activity of each, as assayed by P^{32}-ADP exchange, was the same. Under these conditions any difference in the amount of ADP-C^{14} incorporated must be due to interference by nucleases present in the crude but largely absent from the purified preparation. It may be seen that there was much less incorporation with the crude than with the purified enzyme and that DNA had no effect (except for some inhibition) in either case.

Nature of product: The radioactive nucleotides are incorporated into a product which is acid-insoluble, nondialyzable and, like RNA, remains in the aqueous phase on extraction with phenol. Furthermore, as shown in Table 5 (exp. 1) this product is resistant to DNAase and susceptible to RNAase or alkaline hydrolysis (cf.

TABLE 5

HYDROLYSIS OF LABELED RNA FROM INCUBATION OF P^{32}- OR C^{14}-LABELED ATP AND NON-LABELED GTP, UTP, AND CTP WITH *A. vinelandii* ENZYMES

Experiment no.	Enzyme	Labeled RNA, cpm	Treatment	Fraction assayed	Radioactivity, cpm
1	Zinc-ethanol fraction	P^{32} (5,600)	Hydrolysis	DNAase residue	5,570
				RNAase residue	780
				Alkali residue	0
2	Zinc-ethanol fraction	P^{32} (116,000)	Alkaline hydrolysis	AMP	21,000
				GMP	34,800
				UMP	21,900
				CMP	39,800
3	Extract	C^{14} (280)	Alkaline hydrolysis	Adenosine	43
				AMP	244

P^{32}-labeled RNA was prepared by incubation (20 min at 37°) of the following reaction mixture: Tris buffer, pH 8.5, 600 μmoles; $MgCl_2$, 150 μmoles, inorganic phosphate, 45 μmoles, ATP-α P^{32} (1.8×10^6 cpm/μmole), 3 μmoles; non-labeled GTP, UTP, and CTP, each 15 μmoles; *A. vinelandii* DNA, 1.5 mg; and enzyme (zinc-ethanol fraction of *A. vinelandii*) with 28.8 mg of protein. Final volume, 8.35 ml. The reaction was stopped by addition of 10 ml of 90% phenol and the RNA isolated according to Kirby.[24] 3.5 ml of dialyzed solution were obtained containing, from the absorbancy at 260 mμ, 5.3 μmoles of RNA with a specific radioactivity of 50,000 cpm/μmole. Aliquots (5,600 cpm) of this solution were diluted with carrier *A. vinelandii* RNA (1:40) and used for experiment 1. Another aliquot (116,000 cpm) was diluted with carrier RNA (1:4.3) and used for experiment 2. Other experimental details are given in the section on methods. C^{14}-labeled RNA was isolated by the phenol procedure from a reaction mixture similar to that of experiment 4, Table 2, except for a larger scale (final volume, 4.0 ml). An aliquot with 1900 cpm was used for alkaline hydrolysis in experiment 3 and an aliquot of the neutralized hydrolysate (280 cpm) for electrophoretic separation of the C^{14}-labeled adenosine and 3′(2′) AMP. These were carried out as described in the section on methods. The zinc-ethanol fraction was obtained from the 33–46 ammonium sulfate fraction by precipitation with ethanol at 15% concentration, in the presence of 0.004 *M* zinc acetate, at −6°.

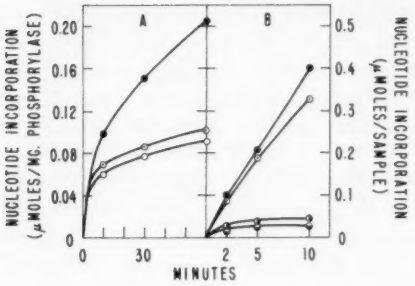

FIG. 3A.—Effect of ribonuclease on RNA synthesis by polynucleotide phosphorylase from ADP-C^{14} and non-labeled GDP, UDP, and CDP. All samples contained (in μmoles/ml) Tris buffer, pH 8.5, 80; $MgCl_2$, 20; ADP-C^{14} (5×10^6 cpm/μmole), 0.4; non-labeled GDP, UDP, and CDP, each 2; and purified *A. vinelandii* polynucleotide phosphorylase (ammonium sulfate-protamine fraction, specific activity 38) with 76 μg of protein. Incubation at 37°. 0.25-ml. aliquots were withdrawn at various time intervals as in Fig. 2. —●—●—●—, no further additions; —○—○—○—, with 2 μg of crystalline pancreatic ribonuclease/ml· —⊙—⊙—⊙—, with 2 μg of ribonuclease and 168 μg of *A. vinelandii* DNA/ml.

FIG. 3B.—Incorporation of nucleotide from ADP-C^{14} into poly AU by crude and purified *A. vinelandii* polynucleotide phosphorylase without and with DNA. Samples contained (in μmoles) in a final volume of 2.0 ml, Tris buffer, pH 8.0, 160; $MgCl_2$, 10; ADP-C^{14} (9×10^6 cpm/μmole), 0.8; UDP, 0.8; and either purified polynucleotide phosphorylase (protamine-ammonium sulfate fraction, specific activity, 38) with 0.095 mg protein (3.6 units), without (—●—●—●—) or with (—⊙—⊙—⊙—) 0.3 mg of *A. vinelandii* DNA or 33–46 ammonium sulfate fraction (specific activity 2.7) with 1.33 mg protein (3.6 polynucleotide phosphorylase units), without (—◑—◑—◑—) or with (—◒—◒—◒—) 0.3 mg. of DNA. Incubation at 30°. 0.25-ml. aliquots were deproteinized with 1.0 ml of 5% trichloroacetic acid at various time intervals.

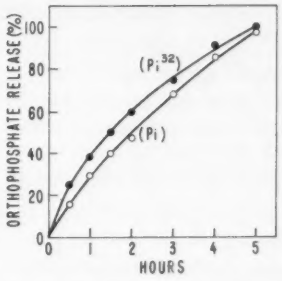

FIG. 4.—Enzymatic hydrolysis of P^{32}-labeled RNA with snake venom. P^{32}-labeled RNA was prepared as described in the legend to Table 5. The hydrolysis reaction mixture, in a final volume of 6.5 ml, contained RNA-P^{32} (diluted with carrier RNA isolated from *A. vinelandii* to give a total radioactivity of 88,200 cpm and a total phosphate content of 24.5 μmoles), 4.5 mg of crude *Crotalus adamanteus* venom, 750 μmoles of glycine buffer, pH 8.5, and 37.5 μmoles of $MgCl_2$. Incubation at 37°. At various time intervals 0.5-ml samples were withdrawn and deproteinized with 0.5 ml of 10% trichloroacetic acid after adding 2 mg of serum albumin to insure complete precipitation of the residual RNA. After standing for 30 min at 0°, the precipitate was removed by centrifugation at 20,000 × *g* for 15 min and suitable aliquots of the supernatant were used for determination of inorganic phosphate[27] and radioactivity. The per cent P_i and P_i^{32} release is based on the total phosphate and radioactivity content of the RNA in the reaction mixture at zero time.

ref. 4). Enzymatic hydrolysis (see Methods) was conducted in a dialysis bag placed in 6 liters of 0.01 *M* Tris–HCl buffer, pH 7.0, with or without 0.01 *M* $MgCl_2$. After 20 hrs at 25° the radioactivity of the bag contents was measured. Alkaline hydrolysis was carried out prior to dialysis. The hydrolysate was then dialyzed for 24 hrs against 6 liters of 0.05 *M* KCl and its radioactivity determined.

Position of incorporated nucleotides in RNA: Labeled RNA prepared with ATP-8-C^{14} was subjected to alkaline hydrolysis followed by electrophoretic separation of the products. Terminal adenylic acid residues are released as adenosine while those inside the RNA chains are liberated as 3′(2′)AMP. As shown in Table 5 (exp. 3) 85 per cent of the AMP-C^{14} incorporated was in non-terminal positions. When RNA prepared from ATP labeled with P^{32} in the α-phosphate was hydrolyzed with alkali all the 3′(2′) nucleotides released were labeled (Table 5, exp. 2). This

indicates that AMP residues were linked to GMP, CMP, and UMP residues in the RNA. In agreement with the results of Weiss,[4] the combined action of the phosphodiesterase and phosphomonoesterase present in snake venom released P_i and P_i^{32} at about the same rate (Fig. 4). This is further proof that the incorporated AMP-C^{14} was predominantly in non-terminal positions and distributed throughout the RNA chains.

Discussion.—The above results show that *L. arabinosus* and *A. vinelandii* contain enzymes distinct from polynucleotide phosphorylase that catalyze the incorporation of nucleotides from ribonucleoside 5′-triphosphates into RNA. They are also distinct from enzymes catalyzing the addition of a few ribonucleotide residues from nucleoside triphosphates to the end of partially degraded chains of amino acid transfer RNA[28] since the incorporated nucleotides are predominantly in non-terminal positions.

The observed requirement for DNA confirms previous reports with other microorganisms.[5, 6] However, little can be said about the mode of action of DNA beyond the statement that its effect cannot be accounted for by an inhibition of nucleases in the enzyme preparations and thus appears to be of a more direct nature. The need for DNA and the fact that all four nucleoside triphosphates are required for incorporation of one of them[29] would be in line with a mechanism for imprinting genetic information in DNA on to RNA, analogous to that of DNA polymerase, whereby DNA would act as a template for determining the order of ribonucleotides in RNA. Further work is required to determine whether this is the case or not, but recent observations on the formation of "hybrid" double-strands between complementary polyribo- and polydeoxyribonucleotides[30, 31] make such a mechanism possible. It is of interest in this connection that heat-denatured T_2 phage DNA and the specific RNA synthesized by the bacterial cell on infection[32] can form "hybrid" double-strands indicating complementarity of nucleotide sequence.[33]

Summary.—Extracts and ammonium sulfate fractions of the microorganisms *Lactobacillus arabinosus* and *Azotobacter vinelandii* bring about an incorporation of labeled nucleotides from ribonucleoside 5′-triphosphates into RNA predominantly in non-terminal positions. Polynucleotide phosphorylase is not involved in this reaction. The presence of ATP, GTP, UTP, and CTP, is required for maximal incorporation of one of them.

Fractionation with ammonium sulfate results in marked decrease of DNA in the active fractions and brings forth a DNA requirement for nucleotide incorporation. The effect of DNA is not due to inhibition of nucleases in the enzyme preparations and appears to be of a more direct nature.

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*ON THE MECHANISM OF INHIBITION OF DEOXYRIBONUCLEIC ACID SYNTHESIS IN EHRLICH ASCITES TUMOR CELLS BY DEOXYADENOSINE IN VITRO**

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It has previously been shown that the incorporation of both C^{14}-formate into DNA[1] thymine and P^{32}-orthophosphate into DNA phosphate of the hypotetraploid strain of Ehrlich ascites tumor cells *in vitro* is strongly inhibited by deoxyadenosine.[2] It was, furthermore, shown that this inhibition is not due to a prevention of the *de novo* synthesis of acid-soluble thymine compounds. The incorporation of P^{32}-orthophosphate into RNA was not significantly inhibited by deoxyadenosine. These observations led to the postulate that deoxyadenosine in a direct or an indirect manner inhibits some specific step in the DNA synthesis of Ehrlich ascites tumor cells *in vitro*.

Prusoff[3] has independently found a similar inhibiting effect of deoxyadenosine on the utilization of formate and of thymidine for the biosynthesis of DNA thymine in Ehrlich ascites tumor cells *in vitro*. Maley and Maley[4] have concluded from recent experiments with labeled pyrimidine nucleosides that deoxyadenosine inhibits DNA synthesis in chick embryos *in vitro*, and Lark[5] has found that the addition of deoxyadenosine to the growth medium of *Alcaligenes fecalis* LB blocks the synthesis of DNA also in these cells.

Among a number of ribosides and deoxyribosides only the synthetically prepared deoxyadenosine 1-N-oxide has been found to have an effect on the DNA synthesis in Ehrlich ascites cells similar to that of deoxyadenosine.[6, 7] It appears, however, that the inhibiting effect of deoxyadenosine is completely absent if deoxyguanosine is added simultaneously to tumor cell suspensions.[6] When only deoxyguanosine is added, the incorporation of P^{32}-orthophosphate may be stimulated up to twofold, and it is possible, therefore, by addition of deoxyadenosine or deoxyguanosine to suspensions of Ehrlich ascites cells, to regulate the rate of DNA synthesis from an 85 to 90 per cent inhibition to a twofold stimulation, respectively. These findings have led to the assumption that deoxyadenosine inhibits DNA synthesis by interfering with the formation of deoxyguanosine or a closely related precursor of DNA.[6]

The metabolism of deoxyadenosine in suspensions of Ehrlich ascites cells has now been investigated. The results have strongly suggested that the effect of this compound is partially or exclusively an indirect one and that a small part of the added deoxyadenosine in the cells is converted to a compound which is the actual inhibitor and which does not penetrate the cell membrane.

Since the cells of the hyperdiploid[8] strain of the Ehrlich ascites tumor may be obtained without contaminating erythrocytes, these have been used in the present experiments in preference to the cells of the hypotetraploid[8] strain previously used.

Materials.—Deoxynucleosides were obtained from the California Foundation for Biochemical Research, Los Angeles, California, and P^{32}-orthophosphate in sterilized isotonic solution from the Radiochemical Centre, Amersham, England.

Cells of the hyperdiploid strain of the Ehrlich ascites tumor were kindly furnished by Dr. G.

Klein, Karolinska Institutet, Stockholm, and they were maintained[9] by transfers made by intraperitoneal injections of ascites fluid into mice of strain DBA. The cells were used 4–5 days after transplantation.

Adenosine deaminase was a highly purified preparation from intestinal mucosa.[10] One unit of enzyme was defined as the amount of enzyme which deaminates 1 μmole of deoxyadenosine per minute at pH 7.5. Xanthine oxidase was prepared as described by Klenow and Emberland.[11] Nucleoside phosphorylase was prepared by the method of Price *et al.*[12]

Methods.—The method for isolation of DNA and the determination of the specific activity of DNA phosphate was essentially as previously described.[2]

Hypoxanthine, deoxyinosine, and deoxyadenosine were determined by enzymatic differential spectrophotometry using the enzymes xanthine oxidase, nucleoside phosphorylase, and adenosine deaminase according to the principle of Kalckar.[13] Samples of the cell suspension were deproteinized with one volume of ice-cold 5 per cent perchloric acid and centrifuged in the cold. A fraction of the supernatant was neutralized with 0.5 *N* KOH, and when $KClO_4$ had precipitated, a known amount of the supernatant was transferred to a quartz cuvette containing 3.0 ml of 0.05 *M* phosphate buffer, pH 7.5. The determination of hypoxanthine, deoxyinosine, and deoxyadenosine was now carried out by successive addition of xanthine oxidase, nucleoside phosphorylase, and adenosine deaminase to the cuvette. By recording the increase in extinction at 292.5 mμ after addition of each of the enzymes, the amount of the corresponding compounds was calculated.[13]

Results.—Evidence for intracellular formation of an inhibitor from deoxyadenosine: The concentration of deoxyadenosine has been determined at time intervals in suspensions of ascites cells incubated with this compound. In the experiment recorded in Figure 1*B*, 50 per cent of the deoxyadenosine had disappeared after about 50 minutes, and after about 3 hours no significant amount was present. Both deoxyinosine and hypoxanthine were formed in the cell suspension. Deoxyinosine was in the beginning formed at a faster rate than hypoxanthine, and deoxyadenosine is, therefore, probably first deaminated to ammonia and deoxyinosine, which is further converted to hypoxanthine and deoxyribose 1-phosphate by a phosphorolytic cleavage. It is, furthermore, seen from this figure that the sum of deoxyadenosine, deoxyinosine, and hypoxanthine decreases slowly until it has reached a level of about 80 per cent of the starting value. It appears from Figure 1*A* that, although almost no deoxyadenosine is present in the cell suspension after about 3 hours of incubation, the inhibition of P^{32} incorporation into DNA is not released even after 5 hours. In another experiment (see Fig. 2) it was found that the inhibiting effect of deoxyadenosine is almost completely abolished when the compound has been preincubated with adenosine deaminase. This further supports a previous finding[2] which suggested that the effect of deoxyadenosine is due to this compound *per se* and not to a contaminating impurity present in the preparations used.

The finding that the inhibition of P^{32} incorporation into DNA is not released even about two hours after deoxyadenosine has disappeared from the cell suspension has led to the postulate that presence of deoxyadenosine in the cells gives rise to the formation of a compound which inhibits DNA synthesis.

This compound would be different from ammonia, deoxyinosine, and conversion products of deoxyinosine, and its inhibiting effect would not be abolished by the addition of adenosine deaminase to the cell suspension. The amount of inhibitor formed from deoxyadenosine would, furthermore, correspond to 20 per cent or less of the added deoxyadenosine. It is, however, not possible from the experiments to exclude the possibility that unconverted deoxyadenosine has an inhibiting effect on DNA synthesis.

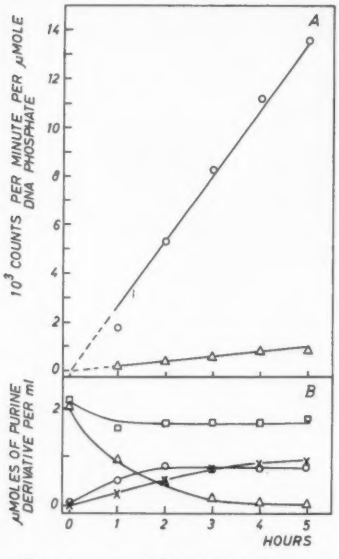

FIG. 1.—Instability of deoxyadenosine in suspensions of Ehrlich ascites tumor cells and persistent inhibition of DNA synthesis. All incubation mixtures contained per ml: 170 mg cells, wet weight; glucose (33 μmoles); sodium succinate (22 μmoles); $P^{32}O_4^{=}$ (about 15 μC) and Tyrode's solution (0.7 ml).

A. Incorporation of P^{32} into DNA. Additions: ○, none; Δ, deoxyadenosine (2.0 μmoles/ml.).

B. Concentration of purine compounds. Aliquots of the cell suspension with added deoxyadenosine were treated with an equal volume of 5 per cent perchloric acid and the supernatant fraction was analyzed as described under *Methods*. Δ, deoxyadenosine; ○, deoxyinosine; ×, hypoxanthine; □, sum of all three purine derivatives.

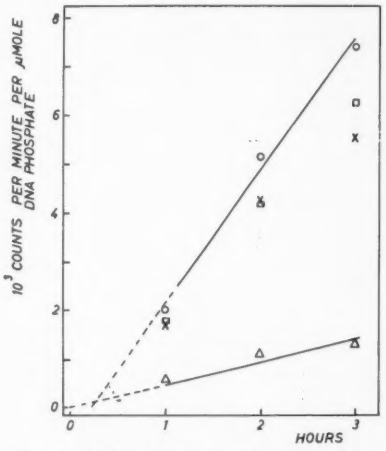

FIG. 2.—Effect of deaminated deoxyadenosine on DNA synthesis. The experimental conditions were as described for Fig. 1. Additions: ○, none; Δ, deoxyadenosine (2 μmoles/ml); □, deoxyinosine (2 μmoles/ml), NH_4OH (2 μmoles/ml) and adenosine deaminase (0.1 unit/ml); ×, deoxyadenosine (2 μmoles/ml) preincubated in Tyrode's solution with adenosine deaminase (0.1 unit/ml) for 1 hour at 37°C.

It was previously observed[6] that the inhibition of DNA synthesis may be abolished when deoxyguanosine is added to the hypotetraploid cells simultaneously with deoxyadenosine. This may now be explained in at least two ways: (1) Deoxyguanosine may prevent the formation of the inhibitor from deoxyadenosine. (2) It may counteract both the effect of the inhibitor formed from deoxyadenosine and a possible inhibiting effect of deoxyadenosine *per se*. In the first case it would not be possible with deoxyguanosine to release the inhibition of cells after preincubation for some time with deoxyadenosine. In the second case, however, it would be possible with deoxyguanosine to overcome the inhibition also after preincubation with deoxyadenosine, provided that the blockage of DNA synthesis in the period of preincubation has not caused other and irreversible changes.

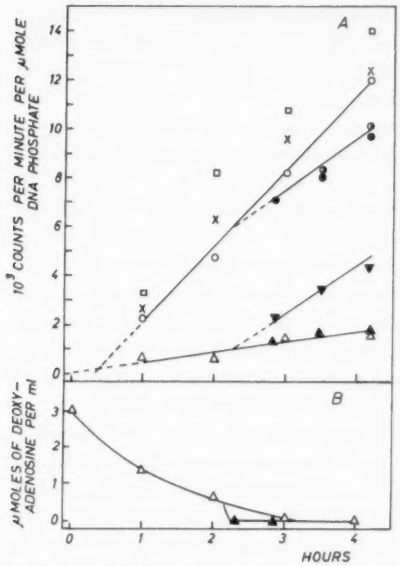

FIG. 3.—Effect of deoxyguanosine on DNA synthesis in ascites tumor cells. The experimental conditions were as described for Fig. 1. *A*. Incorporation of P^{32} into DNA. Additions: flask 1, ○, none; flask 2, Δ, deoxyadenosine (3 μmoles/ml); flask 3, × deoxyguanosine (4 μmoles/ml); flask 4, □, deoxyadenosine (3 μmoles/ml) plus deoxyguanosine (4 μmoles/ml). After incubation for 130 min, 1.25 ml of the mixture in flask 1 were transferred to each of two new flasks (flask 5, ● and 6, ◑) both containing 0.2 units of adenosine deaminase, 7.5 μmoles of deoxyinosine, and 7.5 μmoles of NH_4OH. At the same time 1.25 ml of the mixture of flask 2 were transferred to each of two new flasks (flask 7, ▲ and 8, ▼) both containing 0.2 units of adenosine deaminase. After incubation for further 7 min, 5 μmoles of deoxyguanosine were added to flasks 6 and 8.

B. Concentration of deoxyadenosine; Δ, reaction mixture of flask 2; ▲, reaction mixture of flask 8.

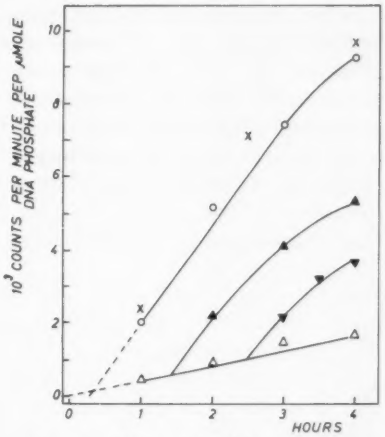

FIG. 4.—Effect of addition of deoxyguanosine to suspensions of Ehrlich ascites tumor cells at various times after deoxyadenosine addition. The experimental conditions were as described for Fig. 1. Additions: ○, none; Δ, deoxyadenosine (4 μmoles/ml). Samples of 1 ml of the mixture containing deoxyadenosine were at the beginning of the experiment (×) and after $1^1/_2$ (▲) and $2^1/_2$ (▼) hours of incubation transferred to new flasks each of which contained 4 μmoles of deoxyguanosine.

The experiment in Figure 3 indicates that also for the hyperdiploid strain of the cells addition of deoxyguanosine at the beginning of the experiment has some stimulating effect on P^{32} incorporation into DNA and that this is even more pronounced in the presence of both deoxyguanosine and deoxyadenosine. The figure shows, furthermore, that the addition of adenosine deaminase to a sample of a cell suspension incubated for 160 minutes with deoxyadenosine does not release the inhibition of P^{32} incorporation into DNA although the concentration of deoxyadenosine rapidly fell to zero. Further addition of deoxyguanosine to such a sample did, however, immediately increase the rate of incorporation to that of the control cells treated in a similar way.

Figure 4 shows again a stimulating effect of deoxyguanosine when added to samples of cells preincubated both for 90 and 150 minutes with deoxyadenosine. In this case, deoxyadenosine was not removed from the samples by addition of adenosine deaminase before the addition of deoxyguanosine.

These findings may suggest, therefore, that the influence of deoxyguanosine is to counteract the effect of the inhibitor formed from deoxyadenosine, rather than to prevent the formation of the inhibitor.

Impermeability of the cell membrane to the postulated inhibitor formed from deoxyadenosine: Experiments have shown that no inhibitor is transferred to control cells with the extracellular fluid of cells that have been incubated with deoxyadenosine and after 100 minutes treated with adenosine deaminase. In another experiment, the acid-soluble fraction was isolated from cells that had been incubated for 180 minutes both under control conditions and in the presence of deoxyadenosine. Figure 5 shows that the incorporation of P^{32}-orthophosphate was inhibited to about the same extent by the two extracts. These findings suggest, therefore, that the inhibitor which is postulated to be formed from deoxyadenosine in the cells does not pass through the cell membrane.

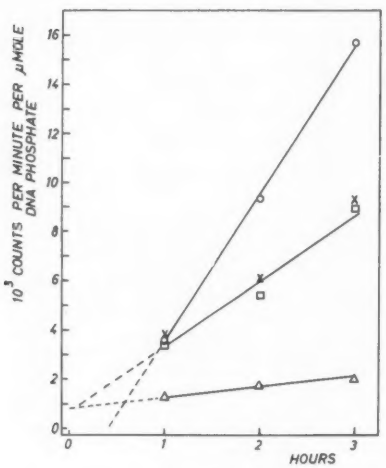

FIG. 5.—Effect of trichloroacetic acid extracts of Ehrlich ascites tumor cells on DNA synthesis in vitro. The experimental conditions were as described for Fig. 1. Additions: ○, none; Δ, deoxyadenosine (2.3 μmoles/ml); ×, 150 μl extract of cells incubated under control conditions; □, 150 μl extract of cells incubated with deoxyadenosine. Extracts of cells were prepared from incubation mixtures composed like those of flasks 1 and 2, respectively. After 180 minutes of incubation the cells were collected by centrifugation, and suspended in about 2 volumes of Tyrode's solution. The suspension was cooled to 0°C and ice-cold trichloroacetic acid was added to give a final concentration of about 4 per cent. The precipitate was removed by centrifugation. The supernatant was extracted 3 times with 5 volumes of ether. The aqueous phase was taken to dryness and dissolved in H_2O (1 ml for each 200 mg of cells present in original incubation mixture).

Discussion.—The experiments indicate that the inhibition of DNA synthesis of Ehrlich ascites tumor cells, by deoxyadenosine, *in vitro*, is partially or exclusively an indirect one. The inhibition of DNA synthesis persists also when deoxyadenosine, after incubation with the cells for about three hours, is removed by deamination. Since deoxyinosine has no effect on DNA synthesis it is postulated that a small part of the added deoxyadenosine in the cells is converted to an inhibitor which is not any more inactivated by adenosine deaminase. It has previously been shown that the inhibiting effect of deoxyadenosine may be prevented by simultaneous addition of deoxyguanosine. Now it has also been found that the effect of the postulated inhibitor may be reversed by deoxyguanosine. The inhibitor appears not to pass through the cell membrane.

It has recently been shown by Munch-Petersen[14] that appreciable amounts of deoxyATP accumulate in the cells incubated with deoxyadenosine. It is possible, therefore, that the inhibitor shown here

to be formed in the cells from deoxyadenosine is identical with deoxyATP. The conclusion that deoxyguanosine presumably counteracts the effect of the inhibitor rather than preventing its formation would be in agreement with this concept since deoxyATP was found[14] to accumulate also when cells were incubated with both deoxyadenosine and deoxyguanosine. Recent experiments in this laboratory have further supported the view that deoxyATP or a closely related compound is the actual inhibitor. It has been shown[15] that addition of adenosine together with deoxyadenosine to a cell suspension not only prevents inhibition of DNA synthesis but also prevents accumulation of detectable amounts of deoxyATP. Furthermore, studies of the kinetics of formation and disappearance of deoxyATP in cells incubated with deoxyadenosine have shown[16] a close correlation between inhibition of DNA synthesis and concentration of deoxyATP.

DNA synthesis in intact cells appears to be inhibited when the deoxyATP concentration increases over a certain level. Formation of deoxyATP in cells incubated with deoxyadenosine may, therefore, interfere with a very efficient mechanism for regulation of the rate of DNA synthesis.

Reichard and his co-workers[17, 18] have recently found that extracts of chick embryo catalyze the reduction of CMP to deoxyCMP and of GMP to deoxyGMP. Both of these reactions appeared[19] to be inhibited by extremely low concentrations of deoxyGTP and of deoxyATP. In the system studied here, deoxyATP or a closely related compound formed in the cells incubated with deoxyadenosine may in a similar way inhibit reduction of GMP to deoxyGMP. The antagonistic effect of deoxyguanosine may be due to the formation of deoxyGMP by direct phosphorylation. In this way the reduction of GMP to deoxyGMP would be bypassed and the level of deoxyguanosine phosphates may be restored to allow normal rate of DNA synthesis.

Summary.—The inhibition of DNA synthesis in the hyperdiploid strain of the Ehrlich ascites tumor cells, by deoxyadenosine, *in vitro*, persists also when deoxyadenosine is removed from the cell suspension by deamination. After the deamination of deoxyadenosine most of the compound can be accounted for as deoxyinosine and hypoxanthine which are both without effect on the DNA synthesis. It has been postulated, therefore, that a small part of the deoxyadenosine added to cell suspensions is converted to an inhibitor of DNA synthesis. The effect of the postulated inhibitor which appears not to pass through the cell membrane may be reversed by added deoxyguanosine. The findings are discussed in relation to recent observations of accumulation of deoxyadenosine triphosphate in cells incubated with deoxyadenosine and to the recently reported formation of deoxyribonucleoside phosphates in cell-free systems.

Valuable technical assistance was given by Miss Hanne Christensen.

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[1] The abbreviations used are: DNA, deoxyribonucleic acid; RNA, ribonucleic acid; deoxyATP, deoxyadenosine triphosphate; CMP, cytidine monophosphate; deoxyCMP, deoxycytidine monophosphate; GMP, guanosine monophosphate; deoxyGMP, deoxyguanosine monophosphate, and deoxyGTP, deoxyguanosine triphosphate.

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RELATION BETWEEN RNA, DNA, AND PROTEIN SYNTHESES IN THE REPLICATING NUCLEUS OF EUPLOTES

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As a repository of genetic specificity DNA must perform two functions, the dissemination of information to the surrounding metabolic system and its own self-replication during growth. At the macromolecular level these two functions possibly proceed by mechanisms that are independent and separate from one another, and as a consequence, they may occur with mutual exclusiveness. Possibly only double-helix DNA can transmit information to the cell and only single-strand DNA is capable of self-replication.[1, 2]

The proposition that information dissemination is carried out through DNA-directed synthesis of RNA is strongly supported by experiment (for review, see Prescott[3]). The most convincing evidence that DNA replication does not involve RNA or RNA synthesis is given by the *in vitro* deoxyribonucleotide polymerase system.[4] Some evidence has been presented, however, that can be interpreted to mean that there may be an intermediate carrier of genetic information between old DNA and the synthesis of new DNA.[5, 6]

The macronucleus of the ciliate protozoan Euplotes has some unique features in the organization of its replication that make it possible to examine visually by autoradiography relations between RNA, DNA, and protein syntheses during nuclear replication. The macronucleus of this cell has the shape of a long narrow ribbon or rod (approximately 140 μ by 7 μ) through which two short-banded structures, the reorganization bands, pass during the last several hours of interphase. Ordinarily, the two bands originate, one at each end of the nucleus, travel through

the nucleus, eventually meet in the center, fuse, and become obliterated very shortly before the amitotic division that accompanies cytokinesis.

Gall[7] recently demonstrated clearly with tritium-labeled thymidine that DNA synthesis takes place in the reorganization band. Fauré-Fremiet *et al.*[8] published a few electron photomicrographs that were helpful in the interpretation of the structural changes in the replicating Euplotes nucleus. We are presenting here experiments dealing with DNA, RNA, and protein synthesis in the Euplotes nucleus that are pertinent to the reputed function of RNA in the replication of DNA.

Materials and Methods.—Euplotes culture: *Euplotes eurystomus* were grown on bacteria (principally *Aerobacter aerogenes*) and *Tetrahymena pyriformis* HSM in dilute lettuce infusion at 24°C. One interdivision interval lasts about 16 hours under these conditions.

Nuclear isolation and autoradiography: Autoradiographs of nuclei labeled with H^3 histidine, uridine, or cytidine were only interpretable when the nuclei were first isolated because of background created by radioactive cytoplasm. With H^3 thymidine labeling there was little or no background of cytoplasmic radioactivity, but isolated nuclei were necessary for the highest possible autoradiographic resolution. Clean isolation of nuclei was accomplished by pipetting living cells into a mixture of glacial acetic acid, lettuce infusion, and 70 per cent alcohol in the proportions of 5:1:1. In this fixative the cells became very fragile and by gently drawing them in and out of a narrow bore-braking pipette the nuclei could be completely freed of cytoplasm. The isolated nuclei were washed in 70 per cent alcohol, affixed to microscope slides, extracted with trichloracetic acid for 5 minutes at 0°C, washed with cold water, and autoradiographed. The autoradiographs were prepared with Kodak NTB2 liquid emulsion and exposure times lasted from 3 to 10 days for H^3 thymidine and 10 to 60 days with H^3 histidine, cytidine, or uridine labeling.

All of the tritium compounds (Schwarz BioResearch) had specific activities of 1.7 to 1.9 C/mM and were added to the culture medium to give final concentrations of 5 to 10 μC/ml.

Digestions: Checks were made on the specificity of isotope incorporations. After drying on slides, nuclei labeled with H^3 uridine or cytidine were treated with (1) 0.02 per cent ribonuclease (Sigma, 5× crystallized) at pH 6.8, 24°C, for two hours, (2) a solution of 0.01 per cent deoxyribonuclease (Sigma, 1× crystallized), 0.005*M* Mg^{++}, pH 6.5, 37°C for 2 hours, or both. A few nuclei from cells fed on H^3 cytidine were treated first with ribonuclease and then with deoxyribonuclease. Extraction with 5 per cent trichloroacetic acid for 5 minutes at 90°C did not remove histidine labeling. Therefore we assumed it had been incorporated into protein.

Staining procedures: Isolated nuclei were stained either with a 0.5 per cent aqueous solution of methyl green (DNA), with mercuric bromphenol blue according to the procedure of Mazia *et al.*[9]—protein staining—with 0.5 per cent aqueous fast green at pH 5.5—protein staining—or with 0.5 per cent aqueous solution of pyronin (RNA).

Interference microscopy: An American Optical-Baker interference microscope was used to estimate the differences in density between regions of the isolated macronucleus by noting the amount of rotation of the analyzer required to give maximum darkness of the various parts.

We are indebted to Mrs. Stella Wilson Perdue for the photography.

Results.—Enzyme digestions: Ribonuclease treatment completely removed incorporated H^3 uridine from all parts of the macronucleus and also removed essentially all incorporated H^3 cytidine from regions that were not involved in DNA synthesis during incorporation. Roughly half of the radioactive cytidine in the DNA synthesis region was removed by ribonuclease digestion, but more than 95 per cent of the activity was removed by digestion with ribonuclease followed by deoxyribonuclease. Incorporated H^3 thymidine was completely removed by digestion with deoxyribonuclease. Thus uridine was incorporated only into RNA, thymidine only into DNA, and cytidine into both.

The reorganization band: The structure of a reorganization band is shown in the phase-contrast photomicrograph (Fig. 1*a*). As described by earlier workers,[10] it consists of two zones, the forward zone (F) or "solution plane" of earlier writers

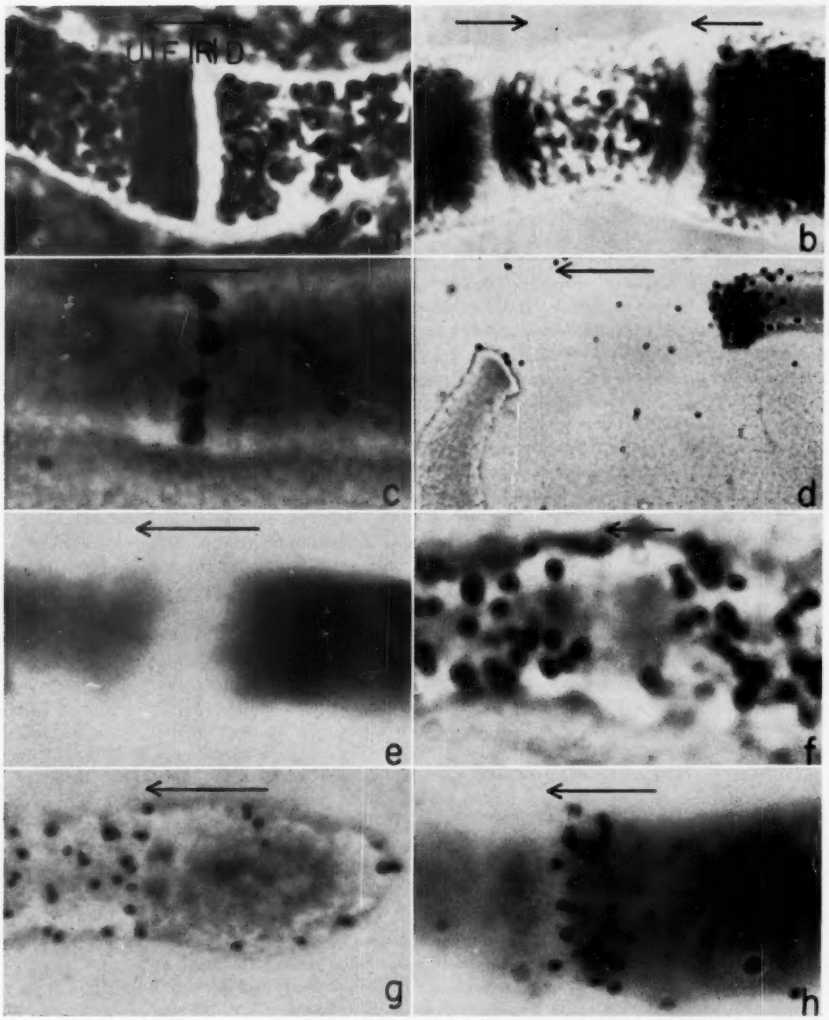

FIG. 1*a*.—Phase contrast photomicrograph of a band and adjacent regions. D, duplicated region; R, rear zone; F, forward zone; U, unduplicated region. The arrows in this and other photographs show the direction of band movement. (2500 ×)

FIG. 1*b*.—Interference photomicrograph showing low density in the rear zone; high, in the forward zone. Two bands approaching each other in the middle of the nucleus. (4000 ×)

(Figs. 1*c*–*h* are bright field photomicrographs.)

FIG. 1*c*.—Two-minute exposure to H^3 thymidine; single row of grains over rear zone. (4000 ×)

FIG. 1*d*.—H^3 thymidine labeling for 20 minutes during which the band moved a short distance; the nucleus is broken at the weak point between the zones with all the label in the rear zone and duplicated region. (1000 ×)

FIG. 1*e*.—Pyronin staining of a replicating nucleus. No stain in either zone. (2500 ×)

FIG. 1*f*.—H^3 uridine labeling for 30 minutes with fixation immediately following. Labeling is present everywhere except in the band. (4000 ×)

FIG. 1*g*.—H^3 uridine labeling for 45 minutes followed by incubation in a non-radioactive medium for $8^1/_4$ hours. The nucleus, therefore, was fixed several hours after exhaustion of the H^3 uridine pulse. No label is present in the band and only slight label in the region through which it has recently traveled. Label is still heavy elsewhere. (2500 ×)

FIG. 1*h*.—H^3 histidine labeling for 40 minutes with fixation immediately following. Slight label is present through most of the nucleus but it is heavy only over the clear band and the region duplicated in the presence of the label. (2500 ×)

and the rear zone (R) or "reconstitution plane." The Feulgen-positive granules of the central, unduplicated part of the nucleus (U) are converted into a relatively dense, homogeneously distributed material at the leading edge of the forward zone. This homogeneous material is transformed abruptly into the less dense, apparently Feulgen-negative material of the rear zone. The boundary between the two zones is a point of mechanical weakness; the isolated macronucleus breaks most frequently at this boundary. The leading portion of the rear zone has a very low density, no more than one-half of that in the forward zone and probably much less, as judged by the interference microscope measurements (Fig. 1*b*). This low density is confirmed by the low amount of DNA and protein staining. The mass in the rear zone increases gradually until it is reorganized, at the trailing edge of the band, into the densely granular material of the duplicated part (D) of the nucleus. The two zones vary in length from nucleus to nucleus, but both are always present. Thus the granular chromatin is first converted into homogeneously distributed but fairly dense material, then undergoes a sudden decrease in density, gradually becomes denser again, and is only then reorganized into discrete granules. The electron microscope pictures of Fauré-Fremiet *et al.*[8] show some of the finer details of these structural changes.

DNA staining and thymidine labeling: The forward zone colors evenly with DNA stains (methyl green and Feulgen). All traces of the organization of DNA into the discrete granules found in the rest of the nucleus is absent. At the rear border of this zone the DNA staining suddenly ends, and no DNA can be demonstrated by methyl green or Feulgen staining in this very first part of the rear zone. DNA staining is visible once more toward the trailing edge of this zone.

Autoradiographs of nuclei isolated from cells that have been exposed to H^3 thymidine for 2 to 5 min prove that all DNA synthesis takes place in the part of the rear zone that cannot be visibly colored with DNA stains (Fig. 1*c*, and 1*d*), that is, in its leading portion. When H^3 thymidine is available to cells for longer and longer periods, the regions of radioactive DNA extend farther and farther behind the zone of synthesis as also shown by Gall.[7] The localization of DNA synthesis in this very short leading portion of the rear zone has been firmly established in autoradiographs of several hundred isolated nuclei labeled for 2 to 60 minutes.

DNA synthesis occurred in the only region in which pre-existing DNA was present in a concentration too low to be demonstrable by staining. The presence of DNA in the region of synthesis was demonstrated, however, by tritium labeling in the previous interdivision interval. Cells engaged in DNA synthesis were exposed to H^3 thymidine for 30 minutes, washed, and grown in nonradioactive nutrient medium until the next DNA duplication cycle was under way. Nuclei isolated from such cells therefore had bands traveling through chromatin with DNA labeled with tritium during the previous replication. Autoradiographs demonstrated that radioactive DNA was present in the DNA synthesis zone, although the silver grain density was always considerably lower over this zone than over any other region of the nucleus. The amount of labeled DNA on the two sides of the moving band was approximately the same. We conclude from this evidence that DNA is present in the synthesis zone but is too dilute to be demonstrated by the staining methods used.

Distribution of RNA and RNA synthesis in the replicating nucleus: Pyronin stains RNA of the Euplotes macronucleus since ribonuclease digestion, which removes all incorporated tritiated uridine, abolishes a measurable amount of dye binding. Some staining is present even after prolonged (10-hrs) digestion with ribonuclease, however. Thus it seems probable that other molecules, perhaps DNA and protein, are stained as well. Pyronin staining is heavy throughout the nucleus except in the reorganization band (Fig. 1*e*). Neither zone of the band binds much stain, suggesting that there is little if any RNA present. The heavier staining in the region through which the bands have already passed (Fig. 1*e*) may be simply the consequence of the greater thickness of these regions. The absence of RNA staining in the bands has been confirmed by Dr. R. C. von Borstel in this laboratory with methyl green and pyronin used in combination. The bright green of the band contrasts sharply with the purple staining in the rest of the nucleus.

Two types of autoradiographic studies of H^3 uridine and cytidine incorporation into RNA of replicating nuclei have been made. In the first type the cells were incubated in the radioactive medium for 5 to 120 minutes, fixed at the end of incubation, and the nuclei isolated for autoradiography. The experiments with cytidine were of limited value because this substance, although excellent for labeling nuclear RNA, also entered metabolic pathways leading to DNA synthesis. Incorporated H^3 uridine, however, was completely removed by ribonuclease digestion. Autoradiographs of the nuclei demonstrated that RNA synthesis was continuous in all regions of the nucleus except in the band (Fig. 1*f*). Judging from the autoradiographs, the rate of RNA synthesis per nuclear surface area was about the same in the replicated and unreplicated regions of the nucleus. No incorporated H^3 cytidine or uridine was ever detected in the forward zone, but H^3 cytidine incorporation was present over the rear zone; this, however, was in DNA because it could be removed from this region by deoxyribonuclease but not by ribonuclease digestion. In uridine experiments no incorporation was demonstrable in either zone. These findings, supported by the pyronin staining results, demonstrate the virtual absence of RNA and of RNA synthesis in either of the zones. Uridine incorporation into RNA was again present in the region just behind the rear zone, showing that RNA synthesis starts again as soon as the reorganization is complete.

In the second type of experiment, cells were exposed to H^3 uridine for 30 to 60 minutes, washed free of the labeled compound, and incubated for periods of 1 to 30 hours in nonradioactive, nutrient medium. Radioactivity continued to enter RNA of the nucleus for 2 to 3 hours after the exogenous source of the labeled precursor had been removed. This radioactivity was supplied by precursor pools and radioactive food organisms ingested during the exposure to the radioactive medium. For several hours after washing, the nuclei continued to have radioactive RNA in all regions except the bands. During this period of hours the bands moved appreciable distances through the nucleus.

Five hours after washing the amount of radioactivity in nuclear RNA began to decrease noticeably. In nuclei fixed 5 to 12 hours after the washing, the bands have had time to move a long distance through the nucleus in the absence of any radioactive precursors of RNA. In these nuclei the RNA in the regions through which the bands have moved after exhaustion of labeled precursor was no longer

labeled (Fig. 1*g*). Such nonradioactive regions, however, strained just as darkly with pyronin as regions containing radioactive RNA. Not all the decrease in radioactivity could be attributed to loss at the band, because even regions remote from the band steadily lost radioactive RNA.

In nuclei fixed after 12 hours, the amount of radioactive RNA per nucleus fell rapidly. By 24 hours almost all nuclei had little or no radioactivity. This was not unexpected because a period of 24 hours is more than sufficient time for all cells to complete one full cell generation.

These findings show that all or nearly all nuclear RNA is either destroyed or transferred to the cytoplasm just ahead of the wave of DNA synthesis. In the region of DNA synthesis, no RNA synthesis takes place, but the latter is resumed once the chromatin is reorganized into granular form. After the supply of labeled precursors for new synthesis is exhausted, radioactive RNA still remains for many hours in regions remote from the bands. This means that RNA, although lost abruptly at the leading edge of the forward zone, is retained for longer periods of time in the rest of the nucleus.

Protein staining and labeling: Isolated, replicating nuclei were stained with the general protein stains, mercuric bromphenol blue and fast green at low pH. With both dyes, staining was present throughout the nucleus except in the rear zone of the band, the region of DNA synthesis. The forward zone strained more intensely than the rest, possibly because of changes in availability of the appropriate reactive groups of the protein. In H^3 histidine labeling studies, radioactivity was incorporated at a very low rate throughout the nucleus but was incorporated at a high rate in the rear zone (Fig. 1*h*). The resolution of the autoradiography was not sufficient to distinguish whether the histidine incorporation took place exactly in the region of DNA synthesis or perhaps slightly behind it. But there is no doubt that both of these syntheses are very closely associated while RNA synthesis does not occur in the region. The autoradiographs were not intense enough to indicate whether or not protein synthesis occurs in the intensely staining forward zone; if it does occur, the rate of synthesis must be very low. Pulse experiments of the type carried out with uridine added very little further information.

Discussion.—The evidence from autoradiography, staining, and interference microscopy shows that the replication of the nucleus, and in particular the synthesis of DNA, proceed according to the following series of events. Exactly at the advancing border of the forward zone, there is a marked change in the organization of the chromatin. The discrete and separate chromatin granules disappear and in the forward zone the chromatin is homogeneously distributed. From studies of this area with the electron microscope, Fauré-Fremiet *et al.*[8] suggest that the chromatin shifts from a tightly coiled or condensed form in the granules to a dispersed, fibrous state in the forward zone. At this point of reorganization there is a sudden loss of all or nearly all RNA and a complete cessation of RNA synthesis. Also at this point protein staining increases but there is very little if any protein synthesis. A second abrupt change in organization of the chromatin occurs at the sharp boundary between the two zones of the band. Here both DNA and protein become temporarily so dilute that no staining can be detected visually, and it is only in this restricted region that DNA synthesis occurs. This synthesis is accompanied by a sharp increase in the rate of protein synthesis, but there is no

detectable RNA synthesis. Finally the chromatin returns to the granular organization according to Fauré-Fremiet *et al.*[8] by coiling of the dispersed fibers, and only then is RNA synthesis resumed.

RNA is formed continuously in the whole macronucleus with the exception of the bands, and all or nearly all of it is eliminated from the nucleus at the time of chromatin reorganization. The data do not allow us to decide whether the RNA is destroyed at this time or transferred to the cytoplasm, but the latter is more likely in view of the evidence[3, 11–13] from other cell types for continuous, substantial transfer from the nucleus to the cytoplasm. If the loss at the leading edge of the band is the result of transfer, then it is much more rapid and complete than elsewhere in the nucleus. RNA in other regions retains activity for a considerable time after labeled precursors are no longer available for the new synthesis. The present data suggest that RNA is lost from the region outside the band but at a lower rate. In any case, with each cycle of DNA replication the nuclear RNA undergoes complete replacement.

The pulse experiment (labeling followed by incubation in nonradioactive medium) indicates that RNA or even breakdown products of it enter the nucleus from the cytoplasm at a very low rate if at all. This follows from the observation that the exhaustion of labeled precursors is followed by a build-up of nonradioactive RNA behind the bands even though the cytoplasm is very heavily labeled.

Protein synthesis occurs in all regions of the nucleus except, perhaps, in the forward zone. There appears to be some sort of change in the proteins in the forward zones, however, since these stain more heavily with certain dyes. This could be a consequence of the change in aggregation of the chromatin or the loss of RNA. The heavy incorporation of histidine in or slightly behind the zone of DNA synthesis may well be the result of newly formed chromosomal protein, especially basic proteins rich in this amino acid. This conclusion is supported by the circumstance that histidine incorporation is high only in the region of thymidine incorporation whereas RNA synthesis, and presumably any concomitant protein synthesis, proceed at uniform rates throughout the regions outside the bands. The work of Alfert,[14] Gall,[7] and Bloch and Godman[15] suggests the simultaneous synthesis of basic protein and DNA. Since RNA synthesis is not resumed in the Euplotes nucleus until chromatin protein has been synthesized, it is possible that this protein may have some role in permitting the release of information from DNA.

The data give no encouragement to the hypothesis that RNA is an intermediate template in DNA synthesis. Rather they indicate that DNA synthesis proceeds in the absence of either RNA or RNA synthesis, at least in the macronucleus of Euplotes. Uchida and Stent[16, 17] have considered alternative ways to account for the data from which the DNA to RNA to DNA hypothesis was originally derived. It seems clear that the hypothesis is not demanded at present by any data although it can be used to give an explanation for certain mutation studies in bacteria.[6] On the contrary, the results of the *in vitro* DNA synthesis experiments and from Euplotes indicate that DNA synthesis can go *in vivo* as well as *in vitro* without an RNA intermediate.

Our first attempts to study synthesis in Euplotes nuclei were prompted by the hypothesis that DNA engaged in its own replication would be incapable of sup-

porting RNA synthesis. The experiments strongly support the hypothesis because mutual exclusiveness of RNA and DNA syntheses has been shown. Several workers[18–21] have found evidence that RNA synthesis is depressed during DNA synthesis. It has been suggested that DNA possibly serves as a template in the synthesis of RNA and by this means genetic specificity is transmitted to the cytoplasm. The two functions of DNA molecules, i.e., self-replication and dissemination of information, may not be capable of proceeding simultaneously in a single DNA molecule. As already mentioned, this temporal separation may reside in the two known configurations of DNA; double helix DNA for release of information and single strand for self-replication. While we have no convincing evidence for single strand DNA in Euplotes nuclei, there are two dramatic reorganizations of DNA at the microscopic level prior to DNA replication.

Summary.—Localization and synthesis of RNA, DNA, and protein have been studied in the replicating nucleus of Euplotes. At the reorganization band, DNA undergoes in succession two marked changes in organization. DNA shifts from a granular state to a dispersed form and finally becomes very dilute; at the time the latter condition is reached, DNA synthesis takes place. Histidine incorporation is heavy only in the region of DNA synthesis. RNA is essentially completely destroyed or eliminated from the nucleus just ahead of the advancing wave of DNA synthesis. Neither RNA synthesis nor RNA are detectable in the zone of DNA synthesis.

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*ON THE PARTICIPATION OF DNA IN RNA BIOSYNTHESIS**

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We have recently described an enzyme system in rat liver nuclei requiring four ribonucleoside triphosphates for the incorporation of ribonucleotides into RNA.[1] Further work with this system showed that the incorporation of ribonucleotides occurred throughout the entire polynucleotide molecule and that this activity disappeared after treatment of the mammalian particles with DNase.[2] Subsequent experiments with DNase-treated rat liver preparations indicated that a partial reactivation of the ribonucleotide-incorporating activity could be achieved with added DNA. In agreement with these observations, Stevens[3] and Hurwitz *et al.*[4] demonstrated the existence of a similar system in extracts from *E. coli*. In addition to requiring the presence of all four ribonucleoside triphosphates in the bacterial system, Hurwitz *et al.* first reported that the addition of DNA was also necessary.

More recently, Weiss and Nakamoto[5] reported the isolation of a similar enzyme system from extracts of the microorganism *M. lysodeikticus* and, using this preparation, demonstrated net synthesis of RNA. In order to obtain some insight into the role played by DNA in this reaction, nearest neighbor sequence analysis and base composition of the newly synthesized RNA were determined. Studies of this type were conducted for a number of different "primer" DNAs and are reported herein.

Using the partially purified *M. lysodeikticus* enzyme, RNA was isolated from the reaction mixture at different times of incubation. Calf thymus or rat liver DNA served as "primer" for this experiment. Analysis of the RNA so prepared suggested that polynucleotides with a similar statistical arrangement of bases were formed at these different reaction times (Table 1). In consequence, it was felt that the nearest neighbor sequence analysis and base composition studies of the RNA synthesized *in vitro* might be meaningful.

Although little information concerning the sequential arrangement of bases in nucleic acid is available, it is probable that the arrangement of deoxynucleotides in DNA differs in DNAs isolated from different sources. If the sequence of ribonucleotides in RNA is influenced by the DNA required for this reaction, then this influence should be reflected in the position of any one ribonucleotide in the RNA chain when various DNA "primers" are used. This relative position may be conveniently determined by preparing RNA enzymatically with one P^{32}-nucleotide, and hydrolyzing the P^{32}-RNA formed with alkali. Since the labeled phosphate is transferred to the adjacent nucleotide under alkaline hydrolysis, one may then examine the relative frequency with which the labeled nucleotide occurs next to a given neighbor.

Examination of the results obtained when calf thymus, rat liver, and different bacterial DNAs serve as "primers" in separate reactions suggests that the relative position of labeled cytidylate in the newly formed RNA is different for the various

"primers" used (Tables 1 and 2). Loss of the terminal nucleotide residue after hydrolysis, contamination of the enzyme or DNA with RNA, or the addition of carrier RNA should have no influence on the results obtained. A complete nearest neighbor study of this type for each labeled ribonucleotide, using a DNA which has

TABLE 1

RATIO OF LABEL IN ALKALINE HYDROLYZED PRODUCTS FROM CMP^{32}-RNA FORMED AT DIFFERENT INCUBATION TIMES

DNA Source	Reaction Time (Minutes)	Total Hydrolyzable Counts	Count Ratio of Isolated Nucleotides* 2′(3′)CMP : 2′(3′)AMP : 2′(3′)GMP : 2′(3′)UMP			
Calf thymus	4	120,000	1.00	1.15	1.01	1.44
Calf thymus	15	218,000	1.00	1.13	0.99	1.51
Rat liver	4	47,400	1.00	1.19	0.80	1.30
Rat liver	15	92,000	1.00	1.13	0.78	1.30

The reaction system contained 9 μmoles of $MnCl_2$, 300 μmoles phosphate buffer of pH 7.5, 0.18 μmoles of CTP^{32} (3×10^7 c.p.m. per micromole), 2.4 μmoles each of ATP, UTP and GTP, 300 μgrams of calf thymus or rat liver DNA and 1.0 mg of *M. lysodeikticus* enzyme. The final volume was 3.0 ml and the vessels were incubated at 25° for the time periods indicated. The reaction was stopped by the addition of 0.60 ml of 3 *N* $HClO_4$ with 4 mg of yeast RNA added as carrier. The precipitate was collected by centrifugation, washed four times by solution in cold 0.10 *N* NaOH and reprecipitation with 0.50 *N* $HClO_4$. The washed residue was hydrolyzed in 0.2 *N* KOH for 18 hours at 37°, acidified with $HClO_4$ to below pH 1 and the acid-soluble fraction saved after centrifugation. This fraction was neutralized and subjected to paper electrophoresis as described previously.[2] The separated nucleotides were assayed for radioactivity. A minimum of 90 per cent of the radioactive material placed on paper was recovered in the nucleotides eluted.

* The total radioactivity found for each isolated mononucleotide was divided by the total counts found for 2′(3′)-cytidylic acid and are the value listed above. Hence, a values of 1.00 indicates a total number of counts equivalent to that found for 2′(3′)-cytidylic acid for that particular experiment.

TABLE 2

RATIO OF LABEL IN ALKALINE HYDROLYZED PRODUCTS FROM CMP^{32}-RNA PREPARED WITH VARIOUS DNAS

DNA Used in Reaction	Count Ratio of Isolated Nucleotides* 2′(3′)CMP : 2′(3′)AMP : 2′(3′)GMP : 2′(3′)UMP			
Pseudomonas	1.00	0.58	0.89	0.68
Serratia	1.00	0.66	1.55	0.80
E. coli	1.00	0.81	0.82	0.89

* The reaction system and the procedure used in these experiments were the same as described in Table 1 except that *Pseudomonas*, *Serratia*, and *E. coli* DNA were used.

been similarly characterized,[6] should give more detailed information as to the role played by DNA in RNA synthesis.

Further information on the participation of DNA in the synthesis of RNA was obtained by determining the base composition of the newly formed ribopolynucleotide. In a series of separate reactions using the microbial enzyme, RNA was prepared *in vitro* when a number of different purified microbial and salmon DNA preparations were present as "primers." The nucleic acid fraction isolated from the reaction mixture was hydrolyzed with alkali, and the products of hydrolysis were separated by paper electrophoresis. No carrier RNA was added at any time. In each of the experiments conducted, a net amount of ribonucleotide material corresponding to about 20 optical density units at 260 mμ was released into the acid-soluble fraction after alkaline hydrolysis. Control incubation vessels in which one nucleotide was omitted during the reaction, but re-added after the reaction had been stopped, gave only 10 per cent of the ultraviolet material obtained with the complete system. The 2′(3′)-nucleoside monophosphates, located on the paper under ultraviolet light after electrophoresis, were eluted and their concentrations determined in a Zeiss spectrophotometer at their respective absorption maxima. The results of these experiments are given in Table 3.

Examination of the base composition of the RNA formed shows a remarkable

TABLE 3

BASE COMPOSITION OF SYNTHESIZED RNA AS COMPARED TO PRIMER DNA
(BASE PROPORTIONS, MOLES PER CENT†)

Base*	*Pseudomonas* DNA	Synthesized RNA Expt. 1	Synthesized RNA Expt. 2	Salmon DNA	Synthesized RNA	*Serratia* DNA	Synthesized RNA	*E. coli* DNA	Synthesized RNA
G	33.0	28.2	35.0	20.8	21.0	29.0	32.6	24.1	23.4
A	18.2	18.3	16.5	29.7	29.0	21.1	18.6	25.4	23.4
C	30.0	32.8	29.5	20.4	23.0	29.0	28.0	25.7	24.8
T(U)	18.8	20.7	19.0	29.1	27.0	20.9	20.8	24.8	28.4
GC	63.0	61.0	64.5	41.2	44.0	58.0	60.6	49.8	48.2
$\frac{A + T(U)}{G + C}$	0.59	0.64	0.55	1.43	1.27	0.72	0.65	1.01	1.07

The complete system contained 30 μmoles of $MnCl_2$, 1.0 mmole of phosphate buffer of pH 7.5, 10 μmoles each of CTP, ATP and GTP, 1.0 mg. of bacterial or salmon DNA and 8 to 10 mg of enzyme (absorbency ratio of 280/260 mμ = 1.54). The reaction was incubated for 1 hour at 25° in a final volume of 10 ml. After incubation, the reaction was treated as described in Table 1, except for the following modifications. No carrier RNA was added. An extraction of the washed acid-insoluble material was made with 10 per cent NaCl, pH 8, at 90° for 30 minutes. The nucleic acid was precipitated twice from the salt extract with two volumes of ethanol. This modification considerably lowered the optical density units released, after alkaline hydrolysis, in control vessels where one nucleotide had been omitted from the reaction mixture.

* Base composition of DNA as reported by Belozersky and Spirin.[11]

† The moles per cent of the bases found in RNA were calculated by assuming that the nucleotides eluted from paper, after electrophoresis, represented 100 per cent recovery. The loss of terminal nucleotide residues by this procedure, as well as endogenous RNA, account in part for the experimental error observed.

similarity to the base composition reported for the respective DNA "primers." *Pseudomonas* and *Serratia* DNA have a relatively high GC mole per cent content, while Salmon DNA has a low GC mole per cent content. Similarly, it can be seen that the GC base content of the RNA formed in the presence of *Pseudomonas* and *Serratia* DNA is high, while the GC content for the RNA found when salmon DNA was used is correspondingly low. When *E. coli* DNA was present in the reaction mixture, the RNA formed had nearly equivalent amounts of each ribonucleotide, which also reflects the base composition of this particular DNA. In each of these experiments, experimental error does not allow for exact comparison between DNA and RNA bases. Examination of experiments 1 and 2 with *Pseudomonas* DNA shows the limit of error incurred. However, the DNAs used here are sufficiently different in composition to exclude experimental error in the interpretation of the results obtained.

Examination of P^{32}-RNA formed after coli infection with phage led Volkin and Astrachan[7] to conclude that the RNA synthesized in the infected cells resembled phage DNA in base ratio. Belozersky and Spirin,[8] after studying DNA and RNA composition in a number of different bacterial species, speculated that a small portion of the cellular RNA might fully correlate in its composition with that of DNA. Yčas and Vincent[9] presented evidence that a metabolically active RNA fraction in yeast had a base composition resembling yeast DNA. More recently, Hall and Spiegelman[10] reported on complex formation between denatured phage DNA and specific RNA. These authors concluded that only T2-DNA complexes with T2-specific RNA since they possess complementary nucleotide sequences. The work described in the present paper agrees with and supports the concept put forth by the above authors.

The *M. lysodeikticus* enzyme used in our experiments will form ribopolynucleotides when mammalian, salmon, sea urchin, bacterial and phage DNA serve as "primers." It is quite possible that in the presence of a specific DNA this enzyme will assemble complementary ribopolynucleotides. However, it should be em-

phasized that we have not yet proved with certainty that the RNA thus synthesized is an exact replica of the "primer" DNA.

Summary.—Experiments are described in which the nearest neighbor to cytidylate and base composition was determined for RNA synthesized by a partially purified bacterial enzyme which requires DNA. The results show that different DNAs alter the position of cytidylate in the newly assembled RNA chain and that the ribopolynucleotide has an average base composition similar to the "primer" DNA used.

The authors are indebted to Drs. E. Peter Geiduschek and Alexander Rich for providing the purified salmon and bacterial DNA.

* This investigation was supported by funds from the Argonne Cancer Research Hospital and from the Joseph and Helen Regenstein Foundation.

† Operated by The University of Chicago for the United States Atomic Energy Commission.

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FURAZAN OXIDES, I. THE STRUCTURE OF BENZOFURAZAN OXIDE

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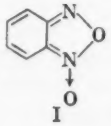
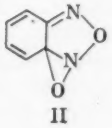
Communicated by John D. Roberts, March 27, 1961

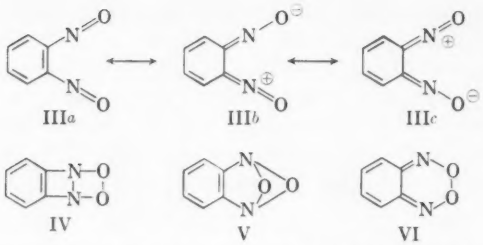
The structure of the compound $C_6H_4N_2O_2$, which may be obtained by treatment of *o*-nitroaniline with alkaline sodium hypochlorite or by pyrolysis of *o*-nitrophenyl azide, has been the subject of controversy since the compound was first recognized in 1894.[1] There is still no general agreement on which of the variety of structures, I–VI, that have been proposed[2] by different workers since that time is correct.



I



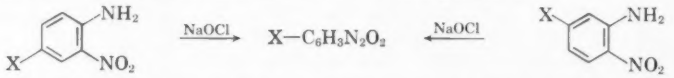
II



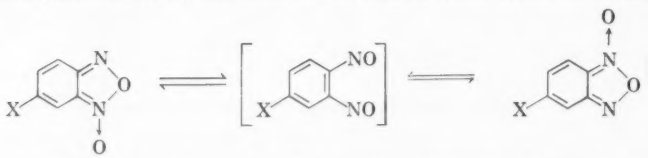
IIIa IIIb IIIc

IV V VI

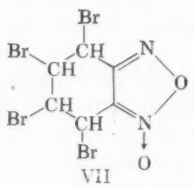
The original suggestion[1] that the compound was *o*-dinitrosobenzene (III*a*) was soon rejected in view of its striking resistance to oxidation[2] by several reagents that readily convert nitroso groups to nitro groups. Recently, however, the *o*-dinitrosobenzene structure has been readvocated,[3] with the modification that the canonical forms III*b* and III*c* make a large contribution to the proposed hybrid. For a brief time the name *ψ*-*o*-dinitrosobenzene was applied to this hybrid structure III,[3] but this notation apparently has been abandoned.[4] Structures V and VI were proposed[2] to account for the lack of properties typical of nitroso compounds and for the fact that only a single compound of formula $X\text{-}C_6H_3N_2O_2$ is obtained from either 2-nitro-4-X-aniline or 2-nitro-5-X-aniline for various groups X:



This apparent equivalence of the two positions across the ring from the nitrogen substituents has been interpreted by several workers to indicate a symmetrical configuration for the N_2O_2 grouping in this type of compound.[5] However, an alternative explanation for this lack of existence of isomers was proposed by Hammick and co-workers,[6] who formulated the parent compound as benzofurazan oxide (I). It was postulated that a facile equilibrium exists between the two isomeric substituted benzofurazan oxides by way of a dinitroso compound as a transient intermediate, such that only the more stable furazan oxide would be isolated:



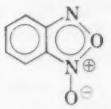
This quinonoid structure, I, was further supported by the demonstration[6] of the addition of bromine to give a tetrabromide, which was formulated as VII. Structure VI, which is also quinonoid, was considered[6] to be unlikely since the compound shows no properties characteristic of peroxides.[2]



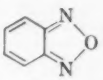
VII

It is now possible to rule out all symmetrical structures such as III, IV, V, and VI on the basis of the 60-megacycle proton NMR spectrum of the compound in either carbon tetrachloride or deuterochloroform solution.* The spectrum obtained in either solvent shows a complicated pattern of proton resonances, which pattern does not have the symmetry expected[7] for the A_2B_2 arrangement of two pairs of equivalent protons.

Two structures remain, I and II, which are consistent with the NMR results. It is felt that structure I is preferable to structure II on theoretical grounds since I should have a considerably larger delocalization energy than II. Structure I contains a system of twelve π electrons delocalized over ten nuclei and may be represented as:



whereas structure II contains the smaller system of eight π electrons delocalized over seven nuclei, with the additional energetic disadvantage that the π-electron system is non-cyclic. Furthermore, the fact that benzofurazan, whose structure is



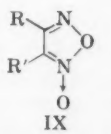
VIII

well-accepted as being VIII, is white (λ_{max} 300.0 mμ, $\epsilon_{300\text{-}0}$ 3330 in 95% ethanol), while the compound here formulated as benzofurazan oxide (I) is yellow (λ_{max} 355.5 mμ, $\epsilon_{355\text{-}5}$ 6960 in 95% ethanol),[8] indicates that the latter contains a larger π-electron system than benzofurazan and not a smaller one as would be the case if structure II were correct.

Thus, it is considered to have been proved that this compound of composition $C_6H_4N_2O_2$ has structure I and may appropriately be named benzofurazan oxide.

Substantiation of this structural assignment is being sought by X-ray crystallographic studies that are now in progress.

The furazan oxide structure has also been demonstrated[9] to be correct for compounds of the open-chain type (IX).



IX

Boyer and Buriks have recently claimed to have demonstrated the existence of two readily interconvertible isomeric forms of this compound $C_6H_4N_2O_2$ of markedly different polarity on the basis of a separation of the pure compound by paper chromatography.[10] However, repetition of this work has shown that this interpretation is invalid. The observed chromatographic separation depends only on the method of impregnation and development of the chromatogram. It was further established that these separations are artifacts by showing that a sample of

o-nitroaniline of high purity gave qualitatively the same chromatographic behavior as I under all conditions investigated.

A detailed presentation of the reconciliation of all known properties of benzofurazan oxide with its structure, I, will be published shortly.

* The spectra were obtained both from Dr. Marjorie C. Caserio of the California Institute of Technology, Pasadena, and independently from Dr. LeRoy F. Johnson of Varian Associates, Palo Alto. This assistance is gratefully acknowledged.

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*A CHEMICAL SYNTHESIS OF ISOMALTOSE**

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Among the naturally occurring oligo- and polysaccharides, a number of the most interesting compounds contain α-linked D-glucose units. The chemical synthesis of all but the simplest α-D-glucopyranosides has proved to be far more difficult than that of the β-D-anomers. The synthesis of a β-D-glucopyranoside link is readily achieved[1] by use of the Koenigs and Knorr reaction[2] in which a tetra-*O*-acyl-α-D-glucopyranosyl halide reacts with a hydroxylic compound, generally in the presence of an acid acceptor. An application of the Koenigs-Knorr reaction to the synthesis of α-D-glucosides has hitherto been of limited success. The known poly-*O*-acyl-β-D-glucopyranosyl halides are either unstable and tend to rearrange to the more stable α-D anomeric form or they react with hydroxylic compounds by a mechanism involving participation of the *trans* 2-*O*-acyl group in the displacement at C-1 leading to products of β-D-configuration.[3] The ability of the protecting group at C-2 to participate in this manner is believed to play an important role in the readiness with which the halides of the β-D-series anomerize. The two such compounds of this series which are relatively stable, namely, 3,4,6-tri-*O*-acetyl-β-D-glucopyranosyl chloride and 3,4,6-tri-*O*-acetyl-2-*O*-(trichloroacetyl)-β-D-glucopyranosyl chloride,[4] are characterized by groups at C-2 which show only a slight tendency to participate.[5, 6] Thus, Hickinbottom[7] was able to prepare, from these halides,

mixtures of the methyl D-glucopyranosides containing considerable proportions of the α-D-anomer. The uncertain course of the reactions of these two halides makes them of dubious value for synthesis of α-D-linked oligosaccharides; indeed, no such syntheses appear to have been reported.

Derivatives of a number of disaccharides containing α-D-glucopyranosyl residues have been synthesized by a variety of chemical methods. These include isomaltose,[8] sucrose,[9] maltose,[10] α,α-trehalose,[11] nigerose,[12, 13] kojibiose,[14] and 2-*O*-α-D-galactopyranosyl-D-glucose.[15] In all cases but the first, the reported yields were low, and for some of the compounds the mechanism by which the α-D-glucosidic bond is formed is not clear. We wish to report herein a novel approach to the synthesis of α-D-linked glucopyranosyl oligosaccharides. In this, use is made of a nitrate ester group which has been shown[16, 17] to be free of the undesirable property of participation in the displacement reaction at a neighboring carbon atom. Carbohydrates containing a hydroxylic function can be nitrated under mild conditions to yield, in most cases, highly crystalline stable products; the nitrate groups may be removed by catalytic reduction[18] when desired. An insertion of a nitrate ester group into the C-2 position of the known[4] 3,4,6-tri-*O*-acetyl-β-D-glucopyranosyl chloride afforded 3,4,6-tri-*O*-acetyl-2-*O*-nitro-β-D-glucopyranosyl chloride. It was anticipated that the reaction of this compound with a hydroxylic derivative should lead to the formation of an α-D-glucopyranosyl bond.

Materials.—"Active" silver carbonate was prepared by a method developed in this Laboratory by Dr. L. H. Klemm. A solution of 16 gm of anhydrous sodium carbonate in 75 ml of water was added dropwise to a mechanically stirred solution of 80 gm of silver nitrate in 200 ml of water. Careful control of the rate of addition ensured that the silver oxide precipitate, which formed where the drops fell, was only transitory. A solution of 10 gm of anhydrous sodium bicarbonate in 125 ml of water was then added in two or three portions; the mixture foamed and the precipitate became yellow. The solid was recovered by filtration and washed at least 12 times by stirring with water and refiltering (the last filtrate should give a negative sodium flame test). The silver carbonate (63 gm) was dried over calcium chloride (not under reduced pressure) in the dark. Best results were obtained if the reactions were carried out in the dark at about 25°.

Chloroform (U. S. P.) was freed of alcohol by washing with water, drying over calcium chloride, and distilling from phosphorus pentoxide. It was kept in the dark in sealed tubes until required. Anhydrous ether was used in the condensation experiments. 1,2,3,4-Tetra-*O*-acetyl-β-D-glucose was prepared by the method of Thompson, Wolfrom, and Inatome.[19]

3,4,6-Tri-O-acetyl-2-O-nitro-β-D-glucopyranosyl chloride: To a mixture of absolute nitric acid, acetic acid, and acetic anhydride (60, 15, and 25 ml, respectively, mixed at 0°), stirred at −40°, was added 9.6 gm of finely powdered 3,4,6-tri-*O*-acetyl-β-D-glucopyranosyl chloride.[4] The reaction mixture was allowed to warm to 0° over a period of 20 min and was then poured onto 2 kg of cracked ice. When the acetic anhydride had hydrolyzed, the white solid was filtered, washed with cold aqueous sodium hydrogen carbonate, then with ice water, and finally dried over phosphorus pentoxide. Crystallization from ethyl acetate-heptane afforded the pure nitrate; yield 9.63 gm (87%), mp 123.5°, $[\alpha]^{22}D$ +14° (*c* 1.0, chloroform); $[\alpha]^{27}D$ +15.2° (*c* 4.0, acetone), constant over 49 hr; $[\alpha]^{20}D$ +12.5° (*c* 0.28, ether) constant over 17 hr.

Anavysis: Calculated for $C_{12}H_{16}ClNO_{10}$: C, 38.10; H, 4.36; N, 3.79; Cl, 9.57. Found: C, 38.21; H, 4.27; N, 3.88; Cl, 9.74.

Methyl 2,3,4,6-Tetra-O-acetyl-α-D-glucopyranoside: A suspension of 3 gm of Drierite ($CaSO_4$ as soluble anhydrite; W. A. Hammond Drierite Co., Xenia, Ohio) and 0.5 gm of silver carbonate in 30 ml of absolute methanol was stirred in the absence of light (foil) for 15 min. To this was added 100 mg of silver perchlorate and 1.00 gm of 3,4,6-tri-*O*-acetyl-2-*O*-nitro-β-D-glucopyranosyl chloride. The reaction mixture was stirred in the dark at room temperature for 3 hr and filtered. The sirupy residue obtained on solvent removal was dissolved in 75 ml of benzene, filtered, and again evaporated. The product was dissolved in 100 ml of ethanol, 50 mg of 10% palladium-on-

charcoal was added, and the mixture was subjected to 45 p.s.i. of hydrogen for 4.5 hr. The sirupy residue obtained on filtration and solvent removal was acetylated with 0.5 gm of sodium acetate and 10 ml of acetic anhydride at 110° for 75 min. The acetylated mixture was cooled and ice-water (250 ml) added. The mixture was stirred to hydrolyze the acetic anhydride and extracted with chloroform. The chloroform layer was washed successively with aqueous sodium hydrogen carbonate and water, dried, and evaporated to a sirup which yielded crystals; yield 846 mg (86%), mp 97–102°. This material was recrystallized from heptane; yield 765 mg (78%), mp 100–101°, undepressed on admixture with authentic methyl tetra-*O*-acetyl-α-D-glucopyranoside, $[\alpha]^{24}D$ +130° (*c* 1.08, chloroform).

When the above reaction was carried out in the absence of silver perchlorate, the yield of product was reduced to 35%.

β-Isomaliose octaacetate: To 100 ml of anhydrous ether was added 3 gm of Drierite, 2 gm of silver carbonate, 100 mg of silver perchlorate, and 2.70 gm (7.5 mM) of 1,2,3,4-tetra-*O*-acetyl-β-D-glucopyranose. To this mixture, stirred in the absence of light, was added 1.00 gm (2.71 mM) of 3,4,6-tri-*O*-acetyl-2-*O*-nitro-β-D-glucopyranosyl chloride. The reaction mixture was stirred for 5 hr at room temperature, after which no chloride ion was detectable. The suspension was filtered through carbon and washed with 100 ml of ether. The combined filtrate and washings were evaporated to a sirup, which was dissolved in 200 ml of abs. ethanol, filtered, and hydrogenated for 50 min at 33 p.s.i. with 10% palladium-on-charcoal catalyst (100 mg). Filtration and evaporation gave a sirup, which was acetylated at 130° for 30 min with sodium acetate and acetic anhydride. The cooled reaction mixture was poured into an excess of ice and water and the mixture was extracted with chloroform. The chloroform extract was washed with aqueous sodium hydrogen carbonate and water, concentrated to a sirup and dissolved in benzene (24 ml). This solution was chromatographed, in two equal parts, on two 25 × 5 cm (diam.) columns of Magnesol-Celite[20] (5:1 by wt). Development with 1% *tert*-butyl alcohol in benzene (1,600 ml), extrusion, and streaking with alkaline permanganate solution resulted in the appearance of two zones. The combined and sectioned zones were extracted with acetone. The extracts were evaporated and the residues were re-evaporated from ethanol solution whereupon they crystallized. β-D-Glucopyranose pentaacetate was obtained on evaporation of the column eluate; yield 2.1 gm, mp and mixed mp 127–129°. The faster-moving band of the column furnished crystalline β-isomaltose octaacetate[21]; yield 1.1 gm (60%), mp 133–143°. Recrystallization afforded pure material; yield 1.01 gm (55%), mp and mixed mp 144–145°, $[\alpha]^{22}D$ +95° (*c* 1, chloroform). The slower-moving band afforded β-gentiobiose octaacetate; yield 112 mg (6%), mp 173–184°. Pure material was obtained on recrystallization from ethanol; yield 91 mg (5%), mp and mixed mp 189–191°.

Much lower yields of the octaacetates of β-isomaltose (9%) and gentiobiose (5%) were obtained on omission of the silver perchlorate, as was reported previously.*

Conversion of 3,4,6-tri-O-acetyl-2-O-nitro-β-D-glucopyranosyl chloride to 3,4,6-tri-O-acetyl-α-D-glucopyranosyl chloride with titanium tetrachloride: To a solution of 1 gm of 3,4,6-tri-*O*-acetyl-2-*O*-nitro-β-D-glucopyranosyl chloride in 20 ml of chloroform was added 3 ml of titanium tetrachloride; an immediate precipitation of a yellow solid occurred. An additional 3 ml of titanium tetrachloride in 20 ml of chloroform was added to the reaction mixture and the whole was heated under reflux for 1 hr. The reaction was terminated by pouring the suspension into 70 ml of ice and water. The mixture was stirred to hydrolyze the excess titanium tetrachloride and was then extracted with chloroform. The chloroform extract was washed successively with water, aqueous sodium hydrogen carbonate solution, and again with water. After solvent removal from the dried chloroform solution, the colorless sirup obtained was crystallized from ethyl acetate–petroleum ether (bp 40–60°); yield 525 mg (52%), mp 86.5–88.5°, $[\alpha]^{26}D$ +181° (*c* 1, chloroform). Recrystallization from the same solvent mixture yielded needles; mp 89–91° undepressed on admixture with authentic 3,4,6-tri-*O*-acetyl-α-D-glucopyranosyl chloride.[4] The material contained chlorine but gave a negative test for nitrate.

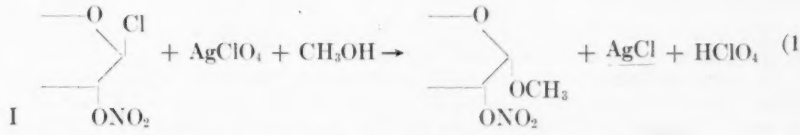
Discussion.—Nitration of 3,4,6-tri-*O*-acetyl-β-D-glucopyranosyl chloride was readily achieved by dissolution in a cold mixture of nitric acid, acetic anhydride, and acetic acid. The crystalline product, isolated in good yield, was 3,4,6-tri-*O*-acetyl-2-*O*-nitro-β-D-glucopyranosyl chloride (I); its optical rotation indicated that

the chloride had not anomerized during the period of reaction. A solution of this substance in acetone or ether exhibited no mutarotation over observation periods of 20 to 50 hours.

An attempt to effect the anomerization of compound I to the presumably more stable α-D-form was made by treating it with titanium tetrachloride in chloroform solution. The product of the reaction was 3,4,6-tri-*O*-acetyl-α-D-glucopyranosyl chloride. Thus, anomerization was effected, but the nitrate ester group was cleaved without deacetylation.

Methanolysis of 3,4,6-tri-*O*-acetyl-2-*O*-nitro-β-D-glucopyranosyl chloride at room temperature in the presence of silver carbonate yielded, after hydrogenolysis and acetylation of the product, methyl tetra-*O*-acetyl-α-D-glucopyranoside (35%) as the only isolable product. A more vigorous reaction occurred when a catalytic quantity of silver perchlorate was added to the reaction mixture, and an improved yield (78%) of methyl tetra-*O*-acetyl-α-D-glucopyranoside was obtained. Bredereck and associates[22] have reported the use of silver perchlorate alone in the synthesis of gentiobiose by the condensation of tetra-*O*-acetyl-α-D-glucopyranosyl bromide with tetra-*O*-acetyl-6-*O*-trityl-β-D-glucopyranose in nitromethane solution.

In a typical Koenigs-Knorr reaction,[1, 2] the rate-limiting step, apparently, is the heterogeneous interaction of the glycosyl halide with the insoluble silver carbonate. In our modification, an excess of silver carbonate was maintained and the perchloric acid, formed on reaction with compound I with the soluble silver perchlorate, acted on the silver carbonate to regenerate the silver perchlorate in solution (2). The driving force of this reaction is probably the concerted exchange, with inversion, of the β-D-chloro atom with the alkoxide through the formation of insoluble silver chloride (1).



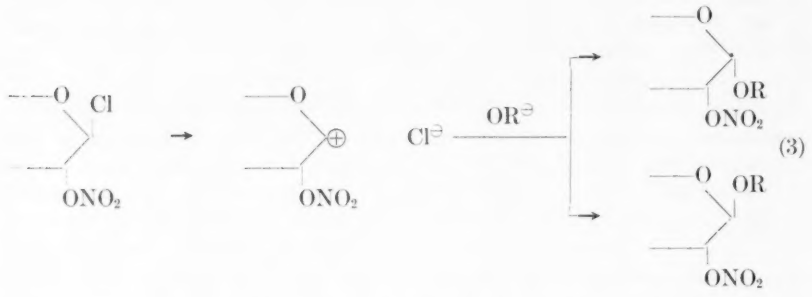
(1)

$$2HClO_4 + Ag_2CO_3 \rightarrow 2AgClO_4 + H_2O + CO_2 \qquad (2)$$

The modification of the Koenigs-Knorr reaction described above was then applied to the synthesis of isomaltose, 6-*O*-α-D-glucopyranosyl-D-glucose. Ether was selected as a suitable inert solvent, and compound I was reacted with an excess of 1,2,3,4-tetra-*O*-acetyl-β-D-glucopyranose under these conditions. The sirupy reaction product was denitrated[18] with hydrogen and palladium, and the reacetylated product was separated chromatographically[23] by silicate column chromatography. β-Isomaltose octaacetate was obtained in 55 per cent yield together with a 5 per cent yield of β-gentiobiose octaacetate. This is an excellent yield of the former substance for this type of reaction. The deacetylation of those octaacetates to the unsubstituted disaccharides is well established.

The origin of the octaacetate of β-gentiobiose, 6-*O*-β-D-glucopyranosyl-D-glucose, is not established. The stability of I in the solvent employed would appear to rule out anomerization of this glycosyl halide. It is probable that its origin lies in a preliminary solvent-assisted dissociation of the glycosyl halide into an ion pair type[24] of reaction (3). Indeed, it is asserted[25, 26] that glycosyl halides react nor-

mally by such a mechanism. The reaction may well occur on the surface of the silver carbonate, and it has been noted[3, 27] that the adsorption of a halogen compound on the polar surface of an insoluble salt facilitates the dissociation of the carbon-halogen bond.



(3)

Summary.—The synthesis of 3,4,6-tri-*O*-acetyl-2-*O*-nitro-β-D-glucopyranosyl chloride, a substance with a non-participating group at C-2, furnishes a new approach to the synthesis of the α-D-glucopyranosyl linkage. The halide is rather unreactive but it is shown that the addition of catalytic amounts of silver perchlorate to the Koenigs-Knorr reaction greatly facilitates the formation of the α-D-glucopyranosyl bond. Methanolysis of the halide with subsequent denitration and reacetylation furnishes methyl α-D-glucopyranoside (tetraacetate) in 78 per cent yield. Reaction with 1,2,3,4-tetra-*O*-acetyl-α-D-glucopyranose furnishes isomaltose (β-octaacetate) in 55 per cent yield together with a small amount (5 per cent) of gentiobiose (β-octaacetate).

* Reported in part: Wolfrom, M. L., and I. C. Gillam, *Science*, **130,** 1424 (1959); and Wolfrom, M. L., *Abstracts Papers A. M. Chem. Soc.*, **139,** 9d (1961).

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A MATHEMATICAL AID IN OPTIMIZING ENGINEERING DESIGNS, II

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The following analysis pertains to a method of finding stationary points of a special type of engineering design function of several variables as described in the previous paper[1] by C. Zener. In this analysis, it will be shown that the method described by Zener is rigorously valid under a set of necessary and sufficient conditions, and those conditions will be described. Further, Zener's analysis stops upon determining the optimum value of their function without specifying the values of the dependent variable at which the extrema occur. Here, the determination of these values will be carried out.

The type of function considered is

$$C(x_1, \ldots, x_{n-1}) = \sum_{j=1}^{n} T_j, \tag{1}$$

where

$$T_j = A_j \prod_{k=1}^{n-1} x_k^{\beta_{kj}}, \tag{2}$$

where A_j and β_{kj} are numbers independent of $(x_1, \ldots, x_{n-1})$. That is, the function C, whose extrema are to be found, is a function of $n - 1$ variables and is expressed as the sum of n terms each of which is a product of powers of the $n - 1$ variables.[2]

The first step in Zener's approach is to construct a set $(\alpha_1, \ldots, \alpha_n)$ such that

$$K \equiv \prod_{j=1}^{n} T_j^{\alpha_j} \tag{3}$$

is independent of $(x_1, \ldots, x_{n-1})$. We shall now derive a means of determining these exponents α_j. From (2),

$$K = \prod_{j=1}^{n} A_j^{\alpha_j} \prod_{k=1}^{n-1} x_k^{\beta_{kj}\alpha_j} = A \prod_{k=1}^{n-1} x_k^{\gamma_k} \tag{4}$$

where

$$A = \prod_{j=1}^{n} A_j^{\alpha_j} \tag{5}$$

and

$$\gamma_k = \sum_{j=1}^{n} \beta_{kj}\alpha_j = 0 \quad (k = 1, \ldots, n - 1), \tag{6}$$

so that

$$K = A. \tag{7}$$

The set of $n - 1$ equations in the n variables $(\alpha_1, \ldots, \alpha_n)$ is not sufficient to determine the α_j. We therefore impose the normalization

$$\sum_{j=1}^{n} \alpha_j = 1 \tag{8}$$

as the nth equation.[3] We then have a set of n inhomogeneous equations, in the n α_j's. The solution to these equations is well known and given by

$$\alpha_j = (-1)^{j-1} \frac{\Delta_j}{\Delta}, \tag{9}$$

where

$$\Delta = \begin{vmatrix} 1 & \cdots & 1 \\ \beta_{11} & & \beta_{1n} \\ \cdot & & \cdot \\ \cdot & & \cdot \\ \cdot & & \cdot \\ \beta_{n-1,1} & \cdots & \beta_{n-1,n} \end{vmatrix} \tag{10}$$

and Δ_j is the determinant arising from that of (8) upon removal of the first row and the jth column.

Then, if there is to be a solution,

$$\Delta \neq 0. \tag{11}$$

Once having the solution for the α_j, one uses the method of Lagrangian multipliers to minimize C with the restraint of (3), namely to find the extrema of

$$f \equiv \sum_{j=1}^{n} T_j - \lambda \prod_{j=1}^{n} T_j^{\alpha_j}. \tag{12}$$

Therefore,

$$\partial f / \partial T_k = 0 = 1 - \lambda K \, \alpha_k / T_k. \tag{13}$$

Note now that if $\alpha_k = 0$, the equation (13) is meaningless. Then in order for the above step of Zener's method to be carried out, one must have

$$\alpha_k \neq 0 \quad (k = 1, \ldots, j), \tag{14}$$

or from (8) and (11),

$$\Delta_j \neq 0. \tag{15}$$

The conditions of equations (11) and (15) are then necessary for Zener's method to be applicable. The significance of the two conditions (11) and (15) will be discussed in the appendix. It will be shown there that (15) is necessary for C to have an extremum, while (11) can be violated with C still having extrema. In the latter case, however, the method described by Zener will not find them.

We shall now show that if the conditions of (11) and (15) are fulfilled, then Zener's method is valid. That is, if the method can be applied at all, it yields the correct values of the extrema of C.

Consider the set of variables $(T_1, \ldots, T_n)$ and the side condition (3). In principle, equation (3) can be solved for any one of the T_j's in terms of the other $n - 1$. Finding the extrema of C with respect to the remaining $n - 1$ T_j's is then possible and will yield the same value as the extrema with respect to $(x_1, \ldots, x_{n-1})$ only if the $n - 1$ T_j's are independent functions of $(x_1, \ldots, x_{n-1})$. This will be true if the Jacobian, J_k, of the remaining T_j's with respect to $(x_1, \ldots, x_{n-1})$ is not identically zero. Assume that it is T_k which is eliminated. Then,

$$J_k = \frac{\partial(T_1, \ldots, T_{k-1}, T_{k+1}, \ldots, T_n)}{\partial(x_1, \ldots, x_{n-1})}.$$

Since from (2),

$$\frac{\partial T_j}{\partial x_i} = \beta_{ij} T_j / x_i, \tag{16}$$

$$J_k = \left(\prod_{j=1}^{n} T_j\right)\left(\prod_{i=1}^{n} \frac{1}{X_i}\right) \Delta_k / T_k. \tag{17}$$

Then $J_k \neq 0$ if and only if $\Delta_k \neq 0$. But this is the condition of (11). We have therefore shown that if condition (11) is fulfilled, the extrema found by considering $(T_1, \ldots, T_{k-1}, T_{k+1}, \ldots, T_n)$ as independent variables are identical to those which would be found by using the original variables.

Therefore, conditions (11) and (15) are necessary if Zener's method is to be possible and sufficient for it to yield the same result as would be obtained by first eliminating one of the T_k.

It is important to note, however, that the ability to satisfy conditions (11) and (15) and to go through the procedure outlined, although yielding a formal solution, may yield an answer which is impossible. In particular, C or some x_i's may be found to be complex. Alternatively, all quantities may turn out to be real, but having nonphysical answers, e.g., negative solutions for x_k where x_k must be positive. In such cases, maxima or minima of the function C will occur at boundary points. For this reason and the fact that one may wish to know the sets $(x_1, \ldots, x_{n-1})$ at which extrema occur anyway, a method to find such sets will now be described.

First consider the solution to (13).

$$T_k = \alpha_k \prod_{k=1}^{n} (A_k/\alpha_k)^{\alpha_k} \tag{18}$$

so that

$$C = \prod_{k=1}^{n} (A_k/\alpha_k)^{\alpha_k}. \tag{19}$$

Now the solution of (9) for $(\alpha_1, \ldots, \alpha_n)$ is a unique set. However, because of the possibility of noninteger values for the α's, the set $(T_1, \ldots, T_n)$ may not be unique. We shall now investigate the effects of this under the restriction that C, $(T_1, \ldots, T_n)$, $(x_1, \ldots, x_{n-1})$ are real.

Let

$$(A_k/\alpha_k) = r_k \exp\{i(\varphi_k + 2n_k\pi)\} \tag{20}$$

where r_k is a real, positive number, and n_k is any integer. Since A_k/α_k is real because A_k and the β_{kj} are real, φ_k can have only the two values 0 and π depending upon the sign of A_k/α_k. Then

$$T_k = \alpha_k R\Phi, \tag{21}$$

$$C = R\Phi, \tag{22}$$

where R is the real, positive number

$$R = \prod_{k=1}^{n} r_k^{\alpha_k} \tag{23}$$

and

$$\Phi = \exp\left\{\sum_{k=1}^{n} (\varphi_k + 2n_k\pi)\alpha_k\right\}. \tag{24}$$

If C and T_k are to be real, Φ must be real. It is possible that this can never happen. In such a case, there would be no maxima or minima except at boundaries. There are only two possible real values of Φ, namely, $+1$ and -1; and both need not be possible simultaneously for a given problem. Whether or not one or both of these values can occur will depend upon whether or not, for the particular values of α_k for a given problem, there exists some set of integers n_k such that the sum in the exponent of (24) can be zero or an integral multiple of π. Thus it is possible that up to two values for the extreme values of C can be found, and these are simply negatives of each other. The situation for the points at which the extrema occur is not as simple.

We now proceed to find the x's. From (2),

$$\ln T_j - \ln A_j = \sum_{k=1}^{n-1} \beta_{kj} \ln x_k \quad (j = 1, \ldots, n). \tag{25}$$

Denote

$$t_j = \ln T_j - \ln A_j, \tag{26}$$

$$X_k = \ln x_k. \tag{27}$$

Then

$$t_j = \sum_{k=1}^{n-1} \beta_{kj} X_k \quad (j = 1, \ldots, n), \tag{28}$$

which is a set of n equations in the $n - 1$ variables $(X_1, \ldots, X_{n-1})$. However, it is simple to show that (22) forms a set of dependent equations. Simply multiply the jth equation of (22) by α_j and sum over all j, i.e., for the left-hand side, from (26), (18), (7), (8), and (5).

$$\sum_{j=1}^{n} \alpha_j t_j = \sum_{j=1}^{n} \alpha_j (\ln T_j - \ln A_j) = 0. \tag{29}$$

The right-hand side becomes by (6)

$$\sum_{j=1}^{n} \sum_{k=1}^{n-1} \beta_{kj} \alpha_j X_k = \sum_{k=1}^{n-1} X_k \sum_{j=1}^{n} \alpha_j \beta_{kj} = 0. \tag{30}$$

Thus the set of n equations (22) are dependent. Assume that the ith equation is dependent on the others. Then we want to solve the set of $n - 1$ equations—

$$t_j = \sum_{k=1}^{n-1} \beta_{kj} X_k \quad (j - 1, \ldots, i - 1, i + 1, \ldots, n). \tag{31}$$

The set of equation (30) will have a solution for the X_k if and only if the determinant of the coefficient of X_k is nonzero. However, inspection immediately shows that this determinant is equal to Δ_j. Then, since $\Delta_j \neq 0$ by hypothesis, (30) has a solution. Note that the solution for the X_k need not be all nonzero.

Let

$$T_j/A_j = w_j \exp\{i(\eta_j + 2m_j\pi)\}, \tag{32}$$

$$x_j = y_j \exp\{i\theta_j\}, \tag{33}$$

where w_j and y_j are real, positive numbers, η_j and θ_j are real numbers ($0 < \eta_j < \pi$, $0 < \theta_j < \pi$), and m_k is any integer. Then by substituting (32) and (33) into (31) and equating real and imaginary parts,

$$\ln w_j = \sum_{k=1}^{n-1} \beta_{kj} \ln y_k \quad (j = 1, \ldots, i - 1, i + 1, \ldots, n); \tag{34}$$

$$\eta_j + 2m_j\pi = \sum_{k=1}^{n-1} \beta_{kj}\theta_k \quad (j = 1, \ldots, i - 1, i + 1, \ldots, n). \tag{35}$$

Now (34) has always a real solution for y_j so that there is no problem in finding the amplitude of the x's. If T_j is restricted to be real, then, since A_j is always real, η_j can have only the values 0 and π depending upon whether the ratio T_j/A_j can be positive or negative. Now if $\eta_j = 0$ for all j then (35) has the solution $\theta_k = 0$, $k = 1, n - 1$, and therefore there is an extremum at $(x_1 = y_1, \ldots, x_{n-1} = y_{n-1})$. On the other hand, if not all $\eta_j = 0$, nonzero solutions to (35) will always exist for the θ_k. In general, (33) will result in complex values for the x_k. However, if there exists some set of m_j such that those θ_k, which from (35) are not zero, have values which are integral multiples of π, then one obtains a real set for $(x_1, \ldots, x_{n-1})$. Since all possible sets of integers m_j are allowed, it is possible that as many as 2^{n-1} possible sets $(x_1, \ldots, x_{n-1})$ can exist. However, the magnitudes of each of the $n - 1$ components will always be the same, but it can conceivably be either positive or negative. Again, the number of such sets is dependent upon the particular problem.

We have thus shown that the procedure outlined by Zener essentially leads to a finite number of sets of values for the original $n-1$ variables at which extrema of C occurs.

Appendix.—In this appendix, a brief discussion of the significance of cases where the method breaks down will be discussed.

1. *Un-normalizable set* $(\alpha_1, \ldots, \alpha_n)$: If it turns out that the condition (3) is met by a set $(\alpha_1, \ldots, \alpha_n)$ such that

$$\sum_{j=1}^{n} \alpha_j = 0, \tag{A1}$$

then it will be impossible to normalize these numbers to satisfy (8); and therefore, in that case,

the method will not be applicable. If such is the case, it will be noted that the condition (8) will be replaced by (A1). Then in order for there to be a solution for $(\alpha_1, \ldots, \alpha_n)$ such that all are nonzero, one must have $\Delta = 0$, where Δ is defined by (10). This case of un-normalizable α_j's is then a result of the more general case $\Delta = 0$ discussed below.

2. *One or more $\alpha_j = 0$:* As has already been noted, not all of $(\alpha_1, \ldots, \alpha_n)$ can be zero. For the sake of simplicity, let us assume that $\alpha_1 = 0$ and $\alpha_n \neq 0$. Then from (3),

$$K = \prod_{j=2}^{n} T_j^{\alpha_j} \tag{A2}$$

independent of $(x_1, \ldots, x_{n-1})$. Then solving (A2) for T_n,

$$T_n = \left[K \left(\prod_{j=2}^{n-1} T_j^{\alpha_j} \right)^{-1} \right]^{1/\alpha_n}. \tag{A3}$$

Let us now make this substitution into (1):

$$C = T_1 + \sum_{j=2}^{n-1} T_j + \left[K \left(\prod_{j=2}^{n-1} T_j^{\alpha_j} \right)^{-1} \right]^{1/\alpha_n}. \tag{A4}$$

One notes therefore that C depends on T_1 only through the linear term. Now by the discussion before and after equations (16) and (17), one sees that the set $(T_1, \ldots, T_{n-1})$ are independent $(\alpha_n \neq 0)$ and, therefore, C can equally well be maximized with respect to them. However, because of the linear dependence on T_1, C does not have a maximum or minimum.

3. *$\Delta = 0$:* Consider the definition of Δ as given in (10). For ease of notation, consider each row of the determinant as a row matrix. Then, if $\Delta = 0$, there must be some set $(b_1, \ldots, b_{n-1})$ such that either Case A or Case B holds

Case A:

$$\sum_{k=1}^{n-1} b_k (\beta_{k1}, \ldots, \beta_{kn}) = (1, \ldots, 1) \tag{A7}$$

If (A7) is to be true, there must be at least one nonzero b_k. For convenience, let $b_1 \neq 0$. Then

$$\beta_{1j} = 1/b_1 - \sum_{k=2}^{n-1} (b_k/b_1)\beta_{kj} \quad (j = 1, \ldots, n), \tag{A8}$$

Then

$$C = x_1^{1/b_1} \sum_{j=1}^{n} A_j \prod_{k=2}^{n-1} (x_1 x_k)^{\beta_{kj}(b_1 - b_k)/b_1}. \tag{A9}$$

If one changes variable to $x'_1 = x_1, x'_2 = x_1 x_2, \ldots, x'_n = x_1 x_n$, and lets $\beta'_{kj} = \beta_{kj}(b_1 - b_k)/b_1$,

$$C = (x'_1)^{1/b_1} \sum_{j=1}^{n} A_j \prod_{k=2}^{n-1} (x'_k)^{\beta'_{kj}}. \tag{A10}$$

Thus, the only dependence of C on x'_1 occurs through the factor $(x'_1)^{1/b_1}$ and therefore C does not have a unique maximum or minimum. For example, if $(1/b_1)$ is an even positive integer, the minimum of C is zero for $x'_1 = 0$ regardless of the value of the other variables. If $(1/b_1)$ is not an even integer, then C can conceivably vary from $+\infty$ to $-\infty$ depending upon the allowed range of x'_1, etc.

Case B:

$$\sum_{k=1}^{n-1} b_k(\beta_{k1}, \ldots, \beta_{kn}) = 0$$

where at least two of the b_k are not zero. Let b_1 be one of the nonzero b_k. Then

$$\beta_{1k} = -\sum_{k=2}^{n-1} (b_k/b_1)\beta_{kj} \quad (j = 1, \ldots, n) \tag{A11}$$

and

$$C = \sum_{j=1}^{n} A_j \prod_{k=2}^{n-1} (x'_k)^{\beta'_{kj}} \tag{A12}$$

where x'_k and β'_{kj} are as defined in Case A above. One notes now that C has become a function of only $n - 2$ variables $(x'_2, \ldots, x'_{n-1})$ consisting of n terms and therefore does not fall into the category of functions considered here. It is only for this case that the method described by Zener will not work, while there still may be extrema. These extrema, however, will not be unique since they will occur at specified values of $(x'_2, \ldots, x'_{n-1})$ which do not uniquely determine a set $(x_1, \ldots, x_{n-1})$.

[1] Zener, Clarence M., these PROCEEDINGS, **47**, 537 (1961).

[2] All the β_{kj} for a given j need not be nonzero. That is, all the $n - 1$ variables need not appear in each term T_j. However, if all the β_{kj} for a given j are equal to zero, that jth term is a constant and should have been dropped from consideration.

[3] It is possible for the sum of the α's to be identically zero and therefore not compatible to the normalization conditions of (7). The significance of this case is discussed in the appendix.

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*A CHARACTERIZATION OF ABELIAN η_α-GROUPS IN TERMS OF THEIR NATURAL VALUATION**

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Let T be a totally ordered set and let $H, K \subset T$. One writes $H < K$ if given $h \in H$ and $k \in K$ then $h < k$. Note, $\emptyset < K$, $H < \emptyset$, and $\emptyset < \emptyset$. Let α be an ordinal number. T is said to be an η_α-set[1] if given $H, K \subset T$ such that $|H| + |K| < \aleph_\alpha$ and $H < K$ then there exists $t \in T$ such that $H < \{t\} < K$. Let $T' \subset T$. T' is said to be cofinal (coinitial) in T if given $t \in T$ there exists $t' \in T'$ such that $t \leq t'$ ($t' \leq t$). Let $W(\alpha)$ denote the set of ordinal numbers less than α. By ω_α is meant the smallest ordinal number for which $|W(\omega_\alpha)| = \aleph_\alpha$. By the *upper (lower) character of* T is meant the smallest number ω_α for which a cofinal (coinitial) subset T' exists of power $\aleph_\alpha$. Let $t \in T$. By the *left (right) character of* t is meant the upper character of $\{x \in T : x < t\}$ (the lower character of $\{x \in T : x > t\}$).[2]

Henceforth, let α be an ordinal number greater than zero. An Abelian group with valuation will be called *α-maximal* if every pseudoconvergent sequence[3] whose length (whose ordinal number) is less than ω_α has a pseudo-limit. Clearly α-maximality is weaker than maximality.[4]

Let G be a totally ordered Abelian group. For $a \in G$, let $X(a)$ be the smallest convex subgroup generated by a. X is a valuation when the convex subgroups are ordered by inclusion. Let P be the range of X and let $P' = P - \{X(0)\}$. Let $a \in G$, $a \neq 0$, and let $Y(a)$ be the largest proper convex subgroup of $X(a)$. Then $G(a) = X(a)/Y(a)$, which will be referred to as a *factor of* G, is isomorphic to a subgroup of

the reals, with which $G(a)$ will be identified. In case $G(a)$ is isomorphic to the integers, let it be identified with the integers.

THEOREM. *G is an η_α-set if and only if (i) the factors of G are either the integers or are the reals, (ii) P' is an η_α-set, and (iii) G is α-maximal.*

Proof: Assume that G is an η_α-set. Let $a \in G$, $a \neq 0$. If $G(a)$ has a smallest positive element, $G(a)$ is the integers. Assume $G(a)$ does not have such an element; then $G(a)$ is dense in the reals. Any real number r can be written as a l.u.b. (g.l.b.) of a countable subset of $G(a)$ that does not contain r. There exists $g \in G$ between pre-images of these two sets in G. Clearly the image of g in $G(a)$ is r, proving (i).

Let $H, K \subset P'$ such that $|H| + |K| < \aleph_\alpha$ and $H < K$. For $p \in P'$, let $t(p)$ be a positive element in $X^{-1}(p)$. If K has no least element, let $g \in G$ such that $Nt(H) < \{g\} < t(K)$, N being the natural numbers. If K has a least element, k_0, and $G(t(k_0))$ is not discrete, we may choose a countable set of positive elements that has 0 as a g.l.b. in $G(t(k_0))$ and let $(g_i)_{i\in N}$ be pre-images of this set in G. Let $g \in G$ such that $Nt(H) < \{g\} < \{g_i\}_{i\in N}$. If k_0 is the least element of K and if $G(t(k_0))$ is discrete, then $X(t(k_0))$ is isomorphic to $Z \times Y(t(k_0))$, ordered lexicographically, Z being the integers. Since the upper character of $Y(t(k_0))$ is at least ω_α, $Nt(H)$ is not cofinal in it. Let $g \in Y(t(k_0))$ such that $Nt(H) < \{g\}$. Then $H < \{X(g)\} < K$ in all cases, proving (ii).

Let $(h_\delta)_{\delta<\epsilon}$ be a pseudo-convergent sequence in G whose length is less than ω_α. Let B be its breadth.[5] Clearly, the lower character of $P - X(B)$ is less than ω_α, thus the point character[6] of $G' = G/B$ is less than ω_α. Choose a strictly alternating Cauchy sequence of length less than ω_α in G'. On choosing pre-images in G of this sequence, we can find a point g between the lower and upper halves of these pre-images. The image of g in G' is clearly the limit of the original sequence; thus G' is Cauchy complete. Clearly the image of $(h_\delta)_{\delta<\epsilon}$ in G' is a Cauchy sequence. Let h' be its limit and let h be a pre-image of h' in G. Then h is a pseudo-limit of $(h_\delta)_{\delta<\epsilon}$ in G, proving (iii).

Conversely, assume that G satisfies (i), (ii), and (iii). Since P' is an η_α-set, the upper, lower, and point characters of G are all at least ω_α. Let Σ be the Dedekind completion of G and assume for a moment that there exists $a \in \Sigma$ both of whose characters are less than ω_α. The mapping $g \to -g$ induces a natural reflection in Σ. Subsets of Σ will be called *symmetric* if they are closed under reflection. There are two semi-group operations, U and L, that can be induced on Σ from G, one by adding upper segments and the other by adding lower segments. Let $b \in \Sigma$ and let $T(b)$ be the smallest convex, symmetric, sub-U-semigroup of Σ containing b. Then $T(b)$ is also closed under L. Let $V(b) = T(b) \cap G$. V is a valuation of Σ into the convex subgroups of G ordered by inclusion.[7] Given $g \in G$, $aUg = aLg$. Let $a + g = aLg$. By Theorem 2.29,[8] $V(a - g) \in P'$ for all $g \in G$. Let $S = \{V(a - g) : g \in G\}$. Applying Theorem 2.29, (i), and Lemma 2.28,[9] one can show that S has no least element. Since the characters of a are less than ω_α, the lower character of S is less than ω_α. Thus, we can choose a pseudo-convergent sequence $(g_\delta)_{\delta<\epsilon}$ of G, of length less than ω_α, such that $(V(a - g_\delta))_{\delta<\epsilon}$ is coinitial in S. Since G is α-maximal there exists $g \in G$ that is a pseudo-limit of this sequence; thus, $\{V(a - g)\} < S$, which is absurd and proves that no such a exists in Σ, proving that G is an η_α-set. Hence, the theorem is proved.

A number of examples can be found which satisfy (i), (ii), and (iii). Let E be an

η_α-set and let $G = R\{E\}$ be the Hahn group[10] with real coefficients and "exponents" in E: i.e., let G be the set of all mappings g of E into R whose support, $s(g)$, is either empty or an anti-well-ordered subset of E, where the addition is point-wise addition and the order is lexicographic. Let $G_\alpha = \{g \in G : |s(g)| < \aleph_\alpha\}$. Let $E_0 \subset E$ and let $H = \{g \in G : g(e) \in Z \text{ if } e \in E_0\}$. Finally, let $H_\alpha = H \cap G_\alpha$. Then G, G_α, H, and H_α satisfy conditions (*i*), (*ii*), and (*iii*) and hence are η_α-sets. Assume that $|E| = \aleph_\alpha$. Then $|G_\alpha| = |H_\alpha| = \aleph_\alpha$. To conclude, let F be the set of formal power series with coefficients in R and exponents in G_α whose supports are of power less than $\aleph_\alpha$. Then F is a real-closed field that is an η_α-set of power $\aleph_\alpha$.[11]

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[1] Hausdorff, F., *Grundzüge der Mengenlehre* (Leipzig, 1914), pp. 180–185.

[2] These definitions of characters are modifications of Hausdorff's, *ibid.*, pp. 142–147.

[3] Ostrowski, A., "Untersuchungen zur arithmetischen Theorie der Körper," *Math. Z.*, **39**, 269–404 (1935), §11.

[4] Krull, W., "Allgemeine Bewertungstheorie," *J. Reine Angew. Math.*, **167**, 160–196 (1932).

[5] Ostrowski, A., *loc. cit.*

[6] In a totally ordered group G, the right and left characters of a point $t \in G$ are the same and equal the right and left characters of 0. The *point character of* G is this ordinal number.

[7] Alling, N. L., "On the existence of real-closed fields that are η_α-sets of power $\aleph_\alpha$" (to appear).

[8] *Ibid.*

[9] *Ibid.*

[10] Hahn, H., "Über die nichtarchimedischen Grössensysteme," *Sitz. der K. Akad. der Wiss. (Vienna)*, **116**, 601–653 (1907).

[11] Alling, N. L., *loc. cit.*

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*INTERCELLULAR TRANSFER OF GAMMA-1 AND GAMMA-2 FORSSMAN HEMOLYSINS**, †

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Avidity, a general term for the firmness of the antigen-antibody union, may play an important role in hemolysis. Within certain limits, the less avid the hemolysin the more efficient it is, because, when hemolysin can dissociate, it can produce serial injuries to red cells provided an excess of complement is present.[1] On the other hand, high avidity, which is associated with a high antigen-binding capacity, results in an inefficient hemolytic activity[2] but is considered desirable in antitoxic and precipitating serums. Avidity of hemolysins can be studied by the inversely related rate of intercellular transfer.

Rabbits repeatedly injected with heated sheep red cell stromata form two Forssman hemolysins. The earliest to reach peak titer occurs in the γ_1 globulins, has a molecular weight of about 1,000,000, and is highly hemolytic, whereas the second one reaches peak titer two weeks or more later, occurs in the γ_2 globulins, has a molecular weight of about 160,000, and is poorly hemolytic.[3, 4] In addition, Forssman antigens from different sources, such as guinea pig kidney, stimulate the

production of various hemolysins with different electrophoretic mobilities, and the Forssman antigen of human type A heated red cell stromata engenders very little of the γ_2 hemolysin even after long continued immunization.[4]

We[2] recently described a method of titrating the net per cent transfer of hemolysins in terms of 50 per cent units from red cell to red cell by using Cr^{51}-labeling of red cells as an indicator. With it, we studied the net intercellular transfer of rabbit hemolysins in normal serums as well as in serums resulting from one injection or reinjection of sheep red cells or heated sheep red cell stromata. Fresh sheep cells with this mild immunization and heated stromata with all types of immunization give rise to the Forssman and not the isophile hemolysin. In general, avidity increased as titer increased, while the per cent transfer decreased as titer increased. Thus, transfer was high (28 to 75%) in normal serums with titers of 15 to 160 units, decreased to values of 3 to 13 per cent by the time peak titers (2,100 to 22,000 units) were attained 7 to 13 days after the initial antigen injection, and then slightly increased to about 15 per cent as hemolysin titers declined (200 to 1,060 units at 4 to 5 months). The sequence after a later antigen injection was similar except that transfer was slightly lower at peak titer (2 to 7 per cent). Furthermore, the hemolysin of both normal and immune serums contained two components, i.e., a nonavid one and an avid one (see *Discussion*).

Subsequent to our work, Goodman and Masaitis[5] essentially confirmed our results using a system which measured the transfer of hemolysin between sensitized sheep red cells and formalized cells—the latter cells unite with antibody and fix complement normally without being lysed. In addition, they studied γ_1 and γ_2 antisheep hemolysins after electrophoretic separation (see *Discussion*).

The present intercellular transfer experiments deal with Forssman antisheep hemolysins which are produced by immunization with human type A red cells, extend our work with sheep cell stromata to repeatedly injected rabbits, and corroborate, except for the degree of transfer, and amplify Goodman and Masaitis' studies on the γ_2 hemolysin.

Materials and Methods.—Previously, we have described the methods of titrating hemolysin in 50% units[6] and of titrating intercellular hemolysin transfer in terms of the 50% units of hemolysin which need to be adsorbed on carrier, i.e., unlabeled, red cells (RBC) to give the net transfer of one 50% unit of hemolysin to unsensitized Cr^{51}-labeled red cells (RAC).[2] The red cells were labeled with Cr^{51} by treating them with $Na_2Cr^{51}O_4$, which was obtained from Abbott Laboratories, North Chicago, Illinois, under the commercial name of "Rachromate." In carrying out the *in vitro* intercellular transfer test, one half ($\simeq 1.6 \times 10^8$ cells) of a 100% unit of sheep red cells (RBC) sensitized with known amounts of a given hemolysin was mixed with an equal quantity of unsensitized Cr^{51}-labeled sheep red cells (RAC). The mixture, after standing for 30 minutes at 37°C to allow the transfer of hemolysin to take place and after the addition of an excess of complement, was incubated for 30 min at 37°C to allow hemolysis to proceed. Then, the amount of Cr^{51} in the supernatant fluid was measured in a γ-ray well-type scintillation counter with a single channel analyzer to determine the amount of hemoglobin liberated from the RAC and, hence, the amount of hemolysin transferred from the sensitized RBC to the unsensitized RAC.

Met peak hemolysin titers during anamnestic responses were obtained by subtracting the titer at the time of the reinjection from the actual peak titer obtained.

Starch zone electrophoresis at pH 8.6 (veronal buffer, 0.1 ionic strength) was carried out on whole serums that had been frozen 2 or 3 months. Just before electrophoresis, each serum was centrifuged 30 minutes at $500 \times g$ to remove any spontaneous precipitate. The method described by Stelos and Talmage[7] was used with the following slight modifications. The starch block was 50 cm long by 18 cm wide by 0.5 cm thick and was usually divided in half lengthwise

to accommodate two serums. Two or 3 ml of each serum was mixed with potato starch and placed in the 40th and 41st segments. This location is designated the starting point in Figures 5 and 6.

After electrophoresis, aliquots of the eluate of each cm segment of a given serum were tested (1) for protein N by the procedure of Lowry *et al.*,[8] (2) for 50% hemolytic units[6] and (3) for per cent transfer.[2] In graphs 5 and 6, these values are expressed per ml eluate.

Experimental Results.—Hemolysin titer and net intercellular transfer were obtained on whole serums from twelve rabbits and from fractions of some of the serums after separation by electrophoresis. Six of the rabbits (1–6) were immunized three times with heated human A red cell stromata (AStr), and the other six rabbits (7–12) were immunized three times with heated sheep red cell stromata (SStr). Each injection was given intravenously and consisted of 1.6×10^9 stromata per kg rabbit. Rabbits 1 through 3 were given one injection of AStr; 35 days later, they were given a reinjection of AStr; and 91 days following the reinjection, they were given four reinjections of AStr during the course of eight days and were killed a week later. Rabbits 4 through 6 were repeatedly injected 13 times thrice weekly for 28 days with AStr; 28 days after the last injection, they were given a second similar immunization of AStr; 52 days after the last of the second immunizing injections, they were given four injections of AStr during eight days; and they were killed a week later. Three exactly similar immunizations were given rabbits 7 through 12 except that these six rabbits were given SStr.

A single injection and reinjection of heated human A red cell siromata (AStr): As may be seen in Figure 1, the initial hemolysin response varied markedly in rabbits 1 through 3. It reached a peak titer of only 105 units in rabbit 1 on 12.3 days, whereas it reached a peak titer of 5,600 units in rabbit 3 on 5.7 days. The per cent transfer of hemolysin was high at first (31–38%) and decreased more at the time of peak titer in rabbit 3 (12%) than in rabbits 1 and 2 (20%). These results are similar to our previous work[2] involving a single injection of SStr, but the response with SStr more often resembled the response in rabbit 3 than in rabbits 1 and 2. As will be seen in the next section, initial immunization with repeated injections of AStr also gave the more intense hemolysin response and the lower per cent transfer encountered in rabbit 3.

The anamnestic response was moderate in rabbits 1 through 3. Peak titer, when adjusted by subtracting the titer at the time of reinjection, was 380 units for rabbit 1 and 2,600 units for rabbit 3. There was little change in the per cent of transfer. In rabbit 1, a plateau of 20 per cent was maintained throughout the 28 days of observation; in rabbit 2, transfer dropped from 30 to 25 per cent; and in rabbit 3, it rose from 15 to 28 per cent. These results differ from the sharp decrease in percent transfer at peak titer in rabbits reinjected once with SStr.[2]

Repeated injections of heated human A red cell stromata (AStr): The initial and anamnestic responses in rabbits 4 through 6 as a result of repeated injections of AStr are shown during the early portion of each immunization in Figure 2. There was an adequate initial hemolysin response and a definite change in per cent transfer in all three rabbits. Thus, peak titers of 13,000 to 47,000 units were attained after induction periods of 2.9 to 3.8 days during antibody rises of 7.7 to 8 days, while the per cent net transfer decreased from normal values of 30 to 45 per cent to values of 13 or 14 per cent. In two of the rabbits, it remained low, and in rabbit 4, it increased to 20 per cent.

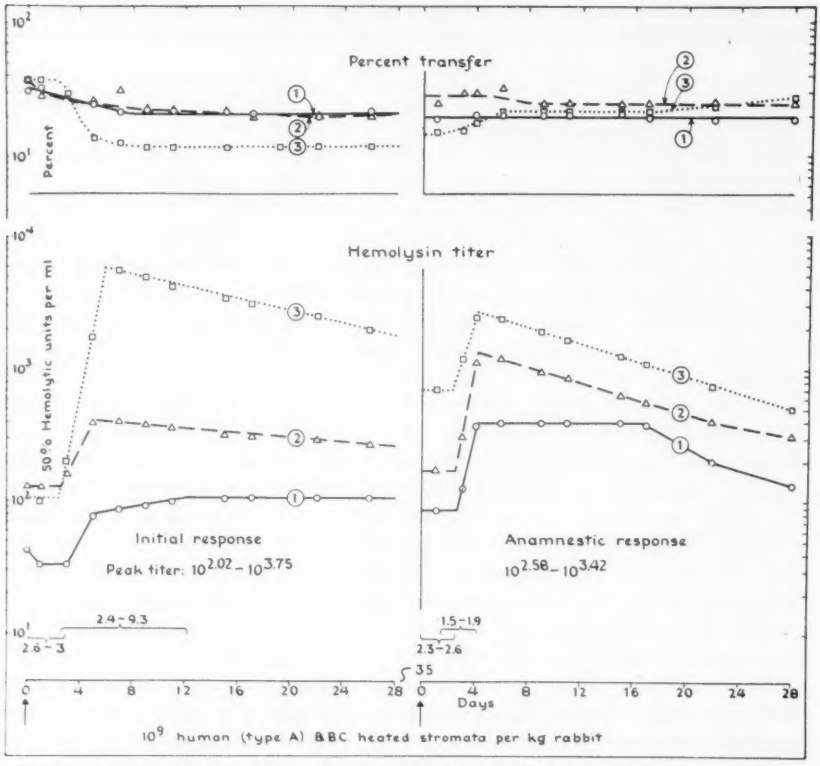

FIG. 1.—Changes in intercellular hemolysin transfer and in hemolytic titer in serums from rabbits 1, 2, and 3 during the initial and anamnestic responses following an intravenous injection and reinjection of heated human A stromata (AStr) as indicated by the arrows.

Hemolysin responses varied, whereas transfer was roughly inversely related to hemolysin titer in the initial response but maintained a plateau during the anamnestic response.

In the first anamnestic response, hemolysin titer and per cent transfer did not change as much as in the initial immunization in spite of the same schedule of injections.

The hemolysin response was slightly more marked in the second than in the first anamnestic immunization but did not equal that of the initial one. Thus, after induction periods of 1.5 to 2 days, titers reached 2,500 to 4,000 units during antibody rises of 3.4 to 9.5 days. The per cent of transfer was little affected. It remained on plateaus of 40 and 29 per cent for rabbits 4 and 5 and rose from 22 to 45 per cent in rabbit 6 during the 14-day period of observation.

Rabbits 1 through 3 had only received one injection and one reinjection of AStr 126 and 91 days previous to their immunization with four injections of AStr. In this second anamnestic response, hemolysin titers of 2,000 to 26,000 units were reached during antibody rises of 7.6 to 8.5 days after induction periods of 1.5 to 2 days, whereas the per cent transfer increased from 31 to 56 per cent in rabbit 1 and maintained plateaus of 39 and 50 per cent in rabbits 2 and 3. Therefore, these anamnestic responses after two single injections of AStr were similar to the anam-

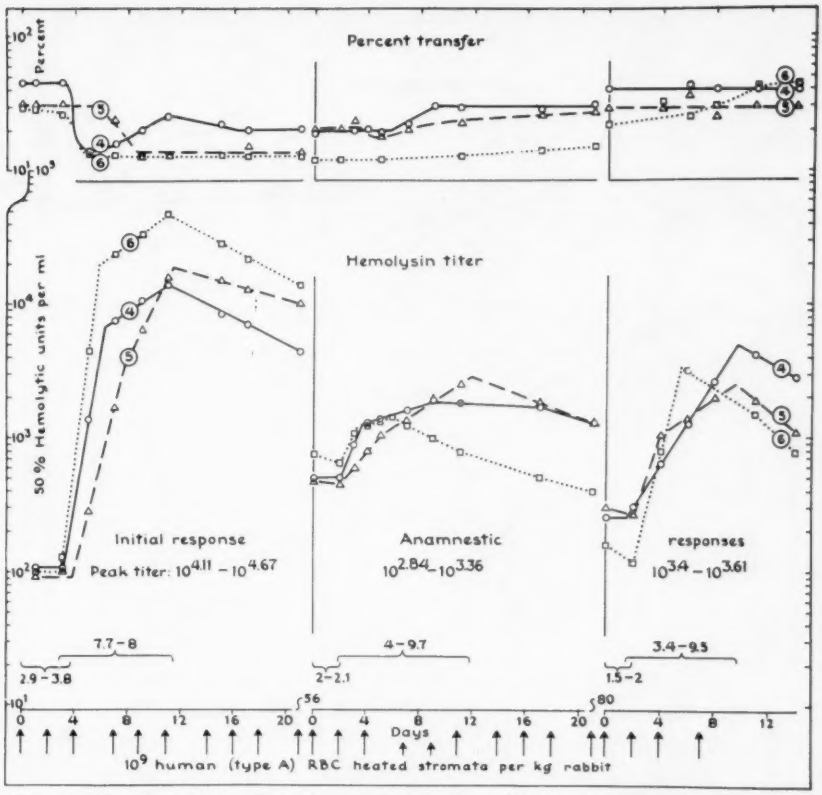

FIG. 2.—Changes in intercellular hemolysin transfer and in hemolytic titer in serums from rabbits 4, 5, and 6 for the first parts of the initial and two anamnestic responses during repeated intravenous injections and reinjections of AStr.

Hemolysin responses were less pronounced and the per cent transfers were higher in the anamnestic responses than in the initial one.

nestic responses after two series of repeated injections of AStr in rabbits 4 through 6.

Repeated injections of heated sheep red cell stromata (SStr): Figures 3 and 4 show the early part of the hemolysin response and per cent transfer during the three immunizations in rabbits 7 through 12. The peaks during the first immunization varied from 45,000 units in rabbit 12 to 93,000 units in rabbit 7 and were reached during antibody rises of 6 to 7.5 days after induction periods of 2.5 to 3 days. The high per cent transfer in all rabbits before injection (50–63%) decreased markedly during the antibody rise, reached its lowest point just before peak titer (4 to 7.9%), and increased slightly thereafter until on the 20th day of immunization it varied from 5.6 to 10 per cent. As compared to rabbits 4 through 6, peak titers in these 6 rabbits were much higher and were reached about a day sooner (although the induction periods were equally long) and the per cent transfer decreased more markedly during the antibody rise.

Hemolysin responses during the second immunization, as compared to the primary one, were not as marked, but peaks were reached after shorter induction

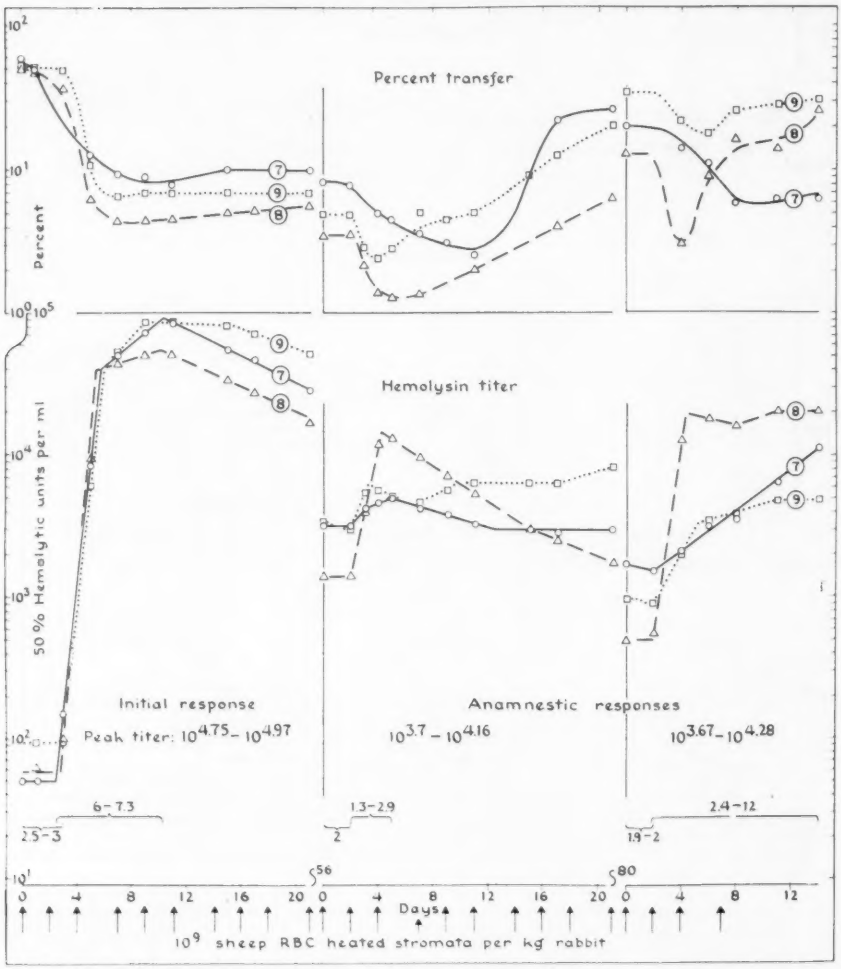

FIG. 3.—Changes in intercellular hemolysin transfer and in hemolytic titer in serums from rabbits 7, 8, and 9 for the first parts of the initial and two anamnestic responses during repeated intravenous injections and reinjections of heated sheep red cell stromata (SStr).

Hemolysin responses were less pronounced and the per cent transfers were higher as immunization was continued.

periods and antibody rises, whereas the per cent transfer decreased to a lower level and rose more rapidly thereafter. As seen in Figures 3 and 4, the per cent transfer varied from 3.6 to 11 on day 56 and decreased at peak titer or shortly thereafter to a low point (1.3 per cent in rabbit 8 to 3.3% in rabbit 11). In rabbit 12, there was a second dip to 2.5 per cent on day 14. The subsequent increase was more marked in rabbits 7, 9, and 11 (12 to 26% on day 20) than in the other 3 rabbits (3 to 7% on day 20). As compared to rabbits 4 through 6, however, peaks were higher and were reached more rapidly, and the per cent transfer decreased more radically.

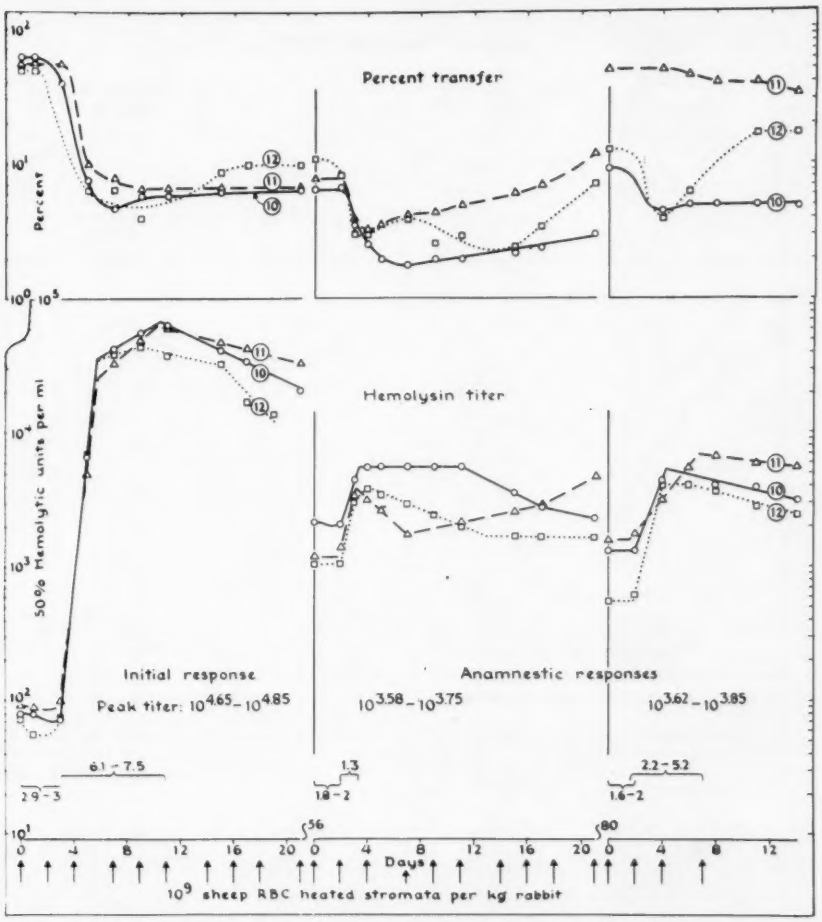

FIG. 4.—Changes in intercellular hemolysin transfer and in hemolytic titer in serums from rabbits 10, 11, and 12 during the same schedule of immunizations with SStr as in Figure 3. Essentially, the same results were obtained as in Figure 3.

At the beginning of the third immunization of rabbits 7 through 12, hemolysin titers varied from 500 to 1,700, and the per cent transfer had reached values of 20 to 50 per cent in three of the rabbits and of 9 to 13 per cent in the other three. The anamnestic hemolysin response was essentially similar to the previous one. The per cent transfer changed more or less characteristically in rabbits 7 and 10, but decreased only briefly in rabbits 8, 9, and 12 and remained practically level in rabbit 11. Changes in transfer with repeated injections of SStr were thus not as pronounced in the third as in the second immunization but were more marked, except for the one in rabbit 11, than during the third immunization with repeated injections of AStr.

Electrophoretic separation of serums during the course of repeated injections of

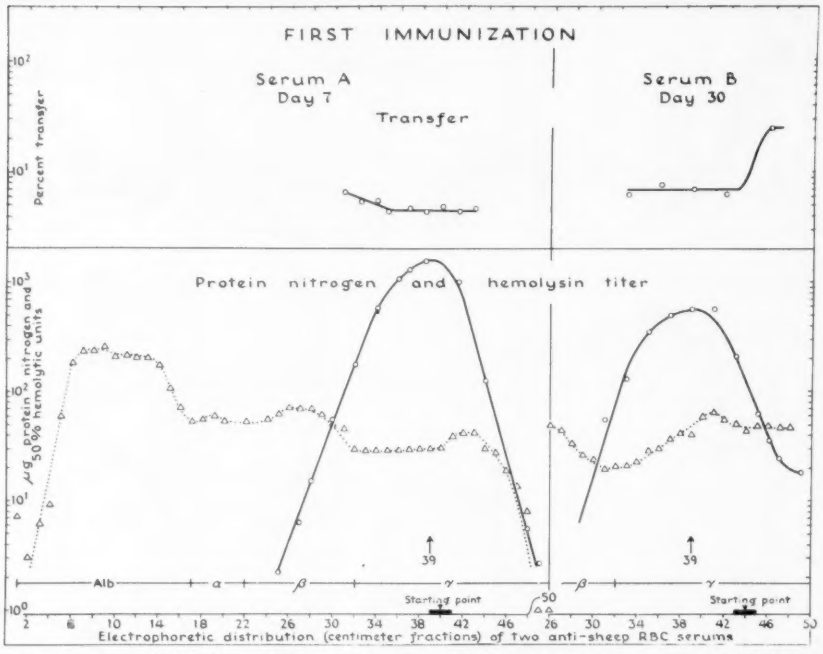

FIG. 5.—Comparison of intercellular hemolysin transfer, hemolytic titer, and protein N (dotted line) in two serums from rabbit 9 separated by starch block zone electrophoresis into 50 one-cm segments.

Serum A contained only γ_1 hemolysin as shown by the fact that hemolysin was normally distributed in segments 26 through 48, whereas serum B contained γ_1 hemolysin as well as a small amount of γ_2 hemolysin as shown by the fact that the hemolysin curve was skewed to the right. Transfer was lower for the γ_1 than for the γ_2 hemolysin.

heated sheep red cell stromata (SStr): Intercellular transfer and hemolytic titer were then studied in electrophoretically separated fractions of whole serums collected at various times during the three immunizations. Five intervals, A through E, were selected. They were: days 7 and 30 of the initial response, days 5 and 32 of the first anamnestic response, and day 14 of the second anamnestic response. These five intervals were tested in serums from rabbits 7, 9, and 11.

The five whole serums from rabbit 9 gave 6.7, 7, 3, 48, and 31 per cent transfer, respectively. After electrophoretic separation, the results are graphed in Figures 5 and 6. Those of serum A are given for centimeter segments of the whole starch block, but those for the other four serums are given only for the segments of the starch blocks containing the globulins of interest.

The 7-day serum A had a well-defined symmetrical hemolysin distribution with a peak at 39 centimeters and titers of less than 10 units below segment 28 and above segment 46. Intercellular transfer for the segments containing hemolysin varied between 4.5 and 7 per cent. These data indicate that the serum was homogeneous and only contained the γ_1 hemolysin with a low per cent transfer.

In the 30-day serum B, hemolysin was present through segment 49, whereas transfer varied from 5 to 7 per cent for segments below 43 and was 25 per cent for a

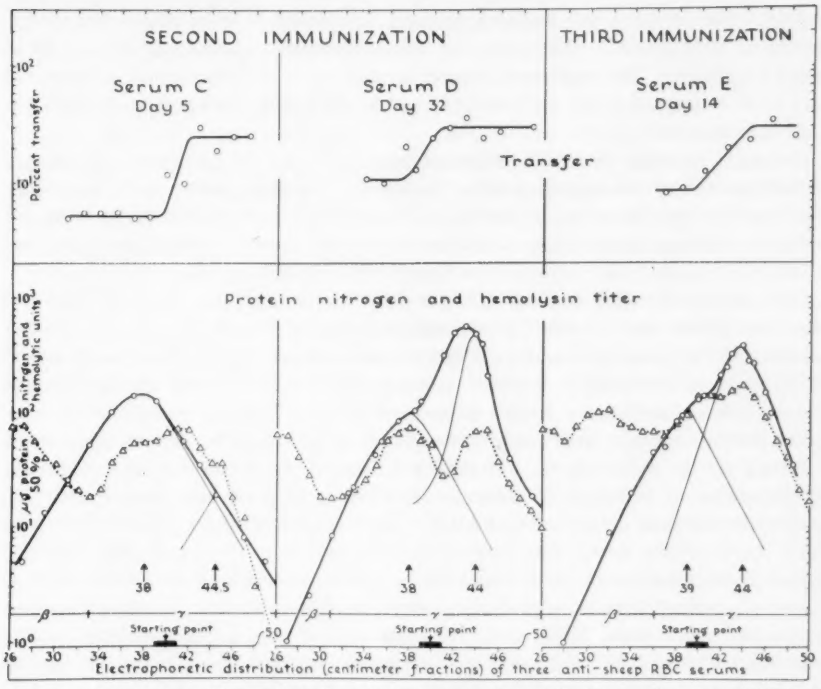

FIG. 6.—Comparison of intercellular hemolysin transfer, hemolytic titer, and protein N (dotted line) in three serums from rabbit 9 separated as in Figure 5.

The γ_2 hemolysin increased in amount as immunization proceeded with a peak at segment 44 and with a higher rate of transfer than the γ_1 hemolysin. Intermediate rates of transfer were obtained in the transitional segments containing both types of hemolysin.

pool of segments 45 through 47 in which hemolysin titers were low. There was a corresponding increase in the protein content of the end segments. These changes reflected the presence of a small amount of γ_2 hemolysin with a high per cent transfer in the γ_2 protein fraction. The amount, however, was not sufficient to raise the per cent transfer of the unfractionated serum.

Serums C, D, and E showed a progressive increase in the γ_2 protein fraction with peak γ_2 hemolysin activity at segment 44. In serum C, the γ_2 hemolysin in segments 44 through 50, when present in sufficient quantities to test, gave from 25 to 31 per cent transfer, while the γ_1 hemolysin in segments 31 through 39 gave from 5 to 9 per cent transfer. In serums D and E, moreover, the γ_2 hemolysin had increased to such an extent that its high per cent transfer masked the low per cent transfer of the γ_1 hemolysin in the whole serums before fractionation.

The foregoing results were substantiated by the data from the five fractionated serums of rabbit 7.

The five whole serums A through E from rabbit 11 gave 8, 7, 4, 37, and 33 per cent transfer. After fractionation, the serums behaved as did those from rabbit 9 except that the γ_2 hemolysin was more clearly evident in serums B and C. In these two serums, there was a definite hemolysin peak at segment 45 and an increase

in transfer from 4 or 5 per cent at segment 41 to a value of 36 to 50 per cent in segments 43 through 48. There was too little hemolysin in segments 49 and 50 to test for transfer. The disproportionately large amount of γ_2 hemolysin in serums D and E, as compared to the γ_1 hemolysin, accounted for the high per cent transfer in the whole serums.

As was expected in view of the reported homogeneity of the antisheep γ_1 Forssman hemolysin,[4] all of the segments of γ_1 hemolysin in a given serum gave essentially the same per cent transfer, as shown in Figure 5. The same statement holds for segments containing only γ_2 hemolysin, as shown in Figure 6. Segments containing mixtures of γ_1 and γ_2 hemolysins gave intermediate values.

The 50 per cent endpoint was used for determining both the hemolysin test and the intercellular transfer test by relating the degree of hemolysis to the dilution of serum in the first case and to the number of hemolysin units needed on the sensitized RBC to cause the transfer of one 50 per cent unit to the unsensitized RAC in the second case. Parallel von Krogh slopes were obtained for these two tests for any given serum and, in general, approximated a 0.4 angle for the γ_1 hemolysin and a 0.2 angle for the γ_2 hemolysin. In addition, some of our serum fractions, especially in the region of segments 39 through 44, which contained both hemolysins, had slopes intermediate between 0.2 and 0.4. These mixtures of the two fractions with their intermediate slopes may account for the fact that Goodman and Masaitis[5] reported an inverse relation between the von Krogh slope and transfer. In their Figure 4, it may thus be seen that 5 serums with a high rate of transfer had an approximate 0.2 slope, 15 serums with a low rate of transfer had a slope varying between 0.38 and 0.48, and 4 serums with an intermediate rate of transfer had an intermediate slope between 0.3 ond 0.35.

Discussion.—The most striking result found in our earlier work[2] was that serums containing normal and immune (mostly γ_1) antisheep hemolysins contained a nonavid component that transferred rapidly and an avid component that transferred slowly. The avid component of normal serums was less avid than the one in immune serums. Moreover, a lapse of time after the first antigen injection and especially after a second antigen injection resulted in an increase in the mean avidity of the whole serum that was largely accounted for by an increase in amount and/or avidity of the avid component. That avidity increased after a second antigen injection was shown by the extremely small amount of transfer in the avid component, even after a two-hour transfer period. The present work indicates that, in addition to such changes, the appearance of γ_2 hemolysin lowers the average avidity.

The present paper extends our previous results[2] on intercellular transfer to the γ_1 Forssman hemolysin made in the rabbit by immunizing with heated human type A red cell stromata. As might be expected, the antiAStr hemolysins behave essentially as do the γ_1 Forssman antiSStr hemolysins. Certain differences, however, were found. For example, as compared to the antiSStr hemolysins, more transfer was obtained in the antiAStr hemolysins after an initial injection of antigen and especially after a second injection. Thus, 12 to 20 per cent and 16 to 31 per cent were the lowest transfers obtained after one injection and one reinjection, respectively, with AStr, whereas 5 to 19 per cent and 1.3 to 4 per cent were reached during similar immunization with SStr (cf. the per cent transfer in the initial and anamnestic responses in Figure 1 with those in Figures 2 and 3 in our earlier work).[2]

Similarly, 12 per cent was the lowest transfer obtained during both immunizations with repeated injections of AStr, whereas 4 to 6 per cent transfer and 2 to 3 per cent were often reached during initial and secondary immunizations, respectively, with SStr (cf. the per cent transfer in Fig. 2 with those in Figs. 3 and 4).

These higher transfers, i.e., lower avidity, of AStr hemolysins cannot be explained by the presence of γ_2 hemolysins because even hyperimmune antiAStr serums contain very little γ_2 hemolysins.[4] They can probably be accounted for by the fact that less antibody is formed after immunizing with AStr than with SStr or possibly by a difference in specificity. In the latter case, a greater avidity might be found if human type A cells were used for the transfer.

It is of interest to point out here that the hemolysin response after peak titer in rabbits 4 through 6, which were repeatedly injected with AStr, declined during all three immunizations and was less intense during the second and third than during the initial immunization. The second and third series of injections were started 28 and 52 days after the last of the preceding injections, respectively. The rabbits thus entered a period of partial immunologic unresponsiveness during their initial immunization (see our work[9] for a discussion of what we originally designated a partial refractory stage during immunization) and had probably not recovered from it when the anamnestic responses were initiated according to unpublished experiments on SStr.

The per cent of transferable antibody, as reported by Goodman and Masaitis,[5] should correspond to our per cent transfer although they used a different system and a 60-minute instead of a 30-minute transfer time. Thus, their range of transfer for 5 to 7 days after a single injection of sheep cells (6 to 20%) is similar to the mean ($10.6 \pm 1.5\%$) previously published by us.[2] On the other hand, other transfer rates do not agree. Thus, 14 to 20 per cent transfer 5 to 9 days after one reinjection of sheep cells appears high as compared to the mean ($4 \pm 0.7\%$) previously published by us.[2] In addition, the low rates of transfer (0 to 4%) for the γ_1 hemolysin and the high rates of transfer (98 to 100%) for the γ_2 hemolysin during late first or second immunizations with repeated injections of sheep cells or stromata are not substantiated by our present results. We obtained 4 to 13 per cent for γ_1 hemolysin and 25 to 50 per cent for γ_2 hemolysin, as exemplified in serums B and D of Figures 5 and 6. In other words, in their electrophoretic fractionation, they obtained an essentially nondissociable γ_1 hemolysin and a readily dissociable γ_2 hemolysin, whereas our nonavid γ_2 hemolysin was only about 4 to 6 times more dissociable than the avid γ_1 hemolysin.

Summary and Conclusions.—The per cent net intercellular transfer of hemolysin from red cell to red cell was studied during the initial and anamnestic hemolysin responses in rabbits given repeated injections of 1.6×10^9 heated sheep red blood cell stromata (SStr) three times a week for a month, or given similar repeated or single injections of heated human A red blood cell stromata (AStr). Intercellular transfer was also studied in some of the antiSStr serums after electrophoretic separation.

In general, during corresponding immunizations, changes in the hemolysin response and in the percent net transfer were greatest with multiple injections of SStr (Figs. 3 and 4), were intermediate with multiple injections of AStr (Fig. 2), and were least with single injections of AStr (Fig. 1). Moreover, with a given antigen, the changes were more marked in the initial immunization than in the

anamnestic responses. Thus, with both antigens, hemolysin titer was, in general, reciprocally related to per cent transfer during the initial immunization, but transfer sometimes remained high at peak titer during the second and especially during the third immunization (Figs. 1–4). This result, with respect to transfer in the rabbits immunized with SStr, was associated with a progressive increase in the γ_2 hemolysin with a high transfer rate and a decrease in the γ_1 hemolysin with a low transfer rate (Figs. 5 and 6). Inasmuch as little or no γ_2 hemolysin has been found by various investigators in response to AStr, the high rates of transfer in the anamnestic responses to AStr were possibly associated with a relative increase in normal hemolysins as production of immune hemolysins decreased.

Transfer was ascertained in terms of the number of 50 per cent units of hemolysin needed to be adsorbed on unlabeled red cells to give the net transfer of one 50 per cent unit of hemolysin to unsensitized Cr^{51}-labeled red cells. The slope of the von Krogh graphs in the tests was usually 0.4 for the γ_1 hemolysin and 0.2 for the γ_2 hemolysin.

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† A preliminary note by the authors has appeared in *Science*, **132**, 1500 (1960).

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NEGATIVE ENTROPY AND PHOTOSYNTHESIS

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As was suggested by E. Schrödinger,[1] the maintenance of the high organization of living beings is due to a continuous influx of negative entropy. Animals get this negative entropy by eating plants (as well as one another) while plants get their negative entropy, along with energy, from the sun's rays. The point is that solar radiation arriving on the earth is not in a state of thermodynamical equilibrium. Indeed, using Wien's formula $\lambda \max T = \text{const.}$ for the maximum of the energy in the spectrum, we obtain $T = 6{,}000°\text{K}$, i.e., the surface temperature of the sun.

On the other hand, since solar radiation is highly diluted while expanding into the surrounding space, its energy density is much smaller when it arrives at the earth, and using the Stefan-Boltzmann formula $e = aT^4$ we get $T = 300°K$, i.e., the surface temperature of the earth.

When this diluted solar radiation falls on the material surface which permits the exchange of energy between different frequencies, an irreversible process is taking place in which the original high-frequency quanta are transformed into a much larger number of the quanta with much lower frequencies. This process results in the increase of the radiation entropy which can be calculated from the quantum statistical theory of radiation. If the surface performing this transformation is made of simple inorganic material, like an iron roof of a house, the total entropy of the system increases and the incident visible light is re-emitted in the form of heat rays.

In the case of the chloroplasts in plant leaves, however, the situation is different, and the increase of the entropy during the irreversible transition of the incident radiation is used to compensate for the decrease of entropy in building organic molecules from the molecules of H_2O and CO_2. Although the mechanics of the photosynthetic process is not yet well understood, purely thermodynamical considerations require that the decrease of entropy per organic molecule formed must not be larger than the increase of entropy of the light quanta participating in the process in their transformation from a high energy inequilibrium state into a low energy state of equilibrium. The purpose of this article is to calculate entropy changes of the incident diluted solar radiation, and to compare them with the measured decrease of entropy in the building process of organic molecules.

Figure 1 shows energy distribution in the spectra of equilibrium (black body) radiation at two temperatures, T_1 and T_2. Suppose that the high temperature radiation, T_1, is diluted to such extent that its integrated energy denisty (the area under the curve A') is equal to the integrated energy density of equilibrium radiation corresponding to the lower temperature T_2 (the area under the curve B). The process taking place when the energy exchange between different frequencies is permitted can be compared to a flow of heat from a reservoir at the temperature T_1 to a cooler reservoir at the temperature T_2. If E is the total

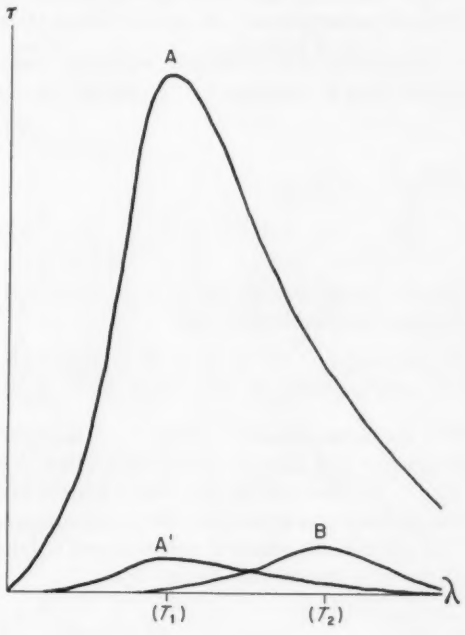

FIG. 1.

amount of energy involved, we may write for the change of entropy:

$$\Delta S \cong E\left(\frac{1}{T_2} - \frac{1}{T_1}\right) \tag{1}$$

The above-given plausibility derivation of this formula can be justified by strict quantum statistical considerations given in the Appendix.

Putting $E = nh\nu$ where n is the number of light quanta participating in the photosynthesis of a single molecule, and neglecting $1/T_{\text{sun}}$ compared with $1/T_{\text{earth}}$, we obtain

$$\Delta S \cong \frac{nh\nu}{T_{\text{earth}}} \tag{2}$$

for the maximum entropy change per molecule formed. Numerically with $\nu \cong 4 \cdot 10^{14}\ \text{sec}^{-1}$ (for red light) and $T_{\text{earth}} \cong 300°\text{K}$, we obtain

$$\Delta S \cong 10^{-14} \cdot n\ \frac{\text{erg}}{1°}\ (\text{per molecule}) = 150 \cdot n\ \frac{\text{cal}}{1°\ \text{mol}} \tag{3}$$

The over-all photochemical reaction:

$$6CO_2 + 6H_2O \rightarrow C_6H_{12}O_6 + 6O_2 \tag{4}$$

requires at least 3 light quanta and corresponds to the entropy change of about 40 cal/1° mol. Thus we conclude that, if the photosynthetic process has at least 10 per cent efficiency in the entropy conversion, the growing of a plant in the sunlight is consistent with the second law of thermodynamics.

Appendix.—The quantum statistical treatment of radiation in temperature equilibrium is well known.[2] In particular this theory leads to the expressions

$$e = aT^4 \tag{5}$$

and

$$s = \frac{4}{3}aT^3 \tag{6}$$

for the energy density and entropy density of black-body radiation, where a is the Stefan-Boltzmann constant,

$$a = \frac{8}{15}\frac{\pi^5 k^4}{(hc)^3}. \tag{7}$$

For the nonequilibrium situation, it is possible to define a temperature[2] for each frequency and also an entropy flux which can be expressed in terms of the energy flux.[3] However for the problem of a black-body sphere radiating into empty space, the problem can be handled directly in a simple manner.

A black-body sphere of radius R_1 will radiate energy E_1 per unit time and entropy S_1 per unit time in amounts given by:

$$E_1 = 4\pi R_1^2 \cdot \frac{1}{4}c \cdot aT_1^4 \tag{8}$$

and

$$S_1 = 4\pi R_1^2 \cdot \frac{1}{4} c \cdot \frac{4}{3} a T_1^3 \tag{9}$$

respectively. In the steady state these same amounts pass through a larger sphere of radius R_2:

$$E_1 = 4\pi R_2^2 \epsilon_2, \tag{10}$$

$$S_1 = 4\pi R_2^2 \sigma_2, \tag{11}$$

where ϵ_2 is the energy flux through the sphere and σ_2 is the corresponding entropy flux. Relation (10) is valid because of conservation of energy. That (11) is valid may be established by noting that there is no mechanism for the production of entropy in the pure radiation field. (Actually the scattering of light by light can increase the entropy, even in empty space, but this fourth-order effect is neglected here.) Another way of establishing (11) is by noting that the transport equation for radiation in space is just the Liouville equation, which is completely reversible with respect to time. The ratio of expressions (11) and (10) is:

$$\frac{\sigma_2}{\epsilon_2} = \frac{4}{3} \cdot \frac{1}{T_1} = \frac{s_1}{e_1}, \tag{12}$$

which shows that the entropy-energy ratio at any distance from the black body corresponds to the *temperature of the black body* emitting the radiation. If we now consider some of the entropy S_1 and energy E_1 initially in the radiation field to be captured in an enclosure and allowed to come to equilibrium, the temperature will become lower than T_1 because of the increase in entropy. In fact, if T_2 is the final temperature and S_2 the final entropy:

$$\frac{S_2}{E_2} = \frac{S_2}{E_1} = \frac{4}{3} \cdot \frac{1}{T_2} \tag{13}$$

and

$$\Delta S = S_2 - S_1 = \frac{4}{3} E_1 \left(\frac{1}{T_2} - \frac{1}{T_1} \right) \tag{14}$$

which, apart from the factor 4/3, is the first displayed equation in the text.

The authors would like to dedicate this paper to the memory of E. Schrödinger and to express to Melvin Calvin, George Salzman, and Harold Walton their thanks for helpful discussions of the subject.

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REMARKS ON LORENTZ CONTRACTION

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There have appeared recently a number of interesting papers[1] concerning the impossibility of seeing or photographing the Lorentz contraction of fast-moving bodies. In these papers, the authors argue that, since the image on the retina of the eye or on the film of the camera is formed by light rays which *enter simultaneously* into the pupil or the lens, it must be distorted. This distortion is due to the fact that these rays *were not emitted simultaneously* from different parts of the moving body at different distances from the observation point. The simple example of a fast-moving cube observed from the side is shown in Figure 1*a*. The rays emitted simultaneously from points A, B, C, and D will clearly take the same time to reach the objective of the camera. But since points E and F are located farther away from the camera, light must be emitted from them somewhat earlier if it has to reach the objective at the same time as light from points A, B, C, and D. The distance by which the cube moves while light covers the distance equal to its side is apparently $v\,(l/c) = l\,(v/c)$; thus, the picture will show the projection of the rear side of the cube with a length $l\,(v/c)$. On the other hand, the face $ABCD$ will be seen as being contracted by the factor $\sqrt{1 - (v^2/c^2)}$ in accordance with Lorentz transformations. Putting $\sin\theta = v/c$; $\cos\theta = \sqrt{1 - (v^2/c^2)}$, we find that on the photograph the cube will appear as preserving its shape but being rotated by angle θ. Considering a sphere inscribed in that cube, we find that a fast-moving sphere will look on the photograph exactly as if it were at rest.

It should be indicated here that the above conclusions depend essentially on the assumption that the object to be photographed is either self-luminous or illuminated by a constant outside source of light. If the photography of a fast-moving cube is performed by a flash-camera or by radar equipment, the situation will be quite different. The parts of the advancing wave which hit the front face of the cube will be reflected back, while those which passed just in front of or just behind it will never have a chance to get back. Thus, the flash camera or radar photographs will definitely show a correct Lorentz contraction.

Another interesting case exists when the moving object is comparatively flat and is moving along an observable trajector like a bicycle (Fig. 1*b*) riding along the curb of a street.[2] In this case, observing the motion of the lower points of the two wheels which always follow the curb, we cannot possibly say that the bicycle is rotated. True, the license plate and the handle bar will look bent out of their normal position, but this deformation could be ascribed to a traffic accident. In the case of a streetcar running along a straight track,[3] the situation is more difficult, and the fact that the rear of the car can easily be seen while all the wheels follow the track cannot be explained in any normal way except by assuming that the entire car suffered an unusual sheer deformation.

It follows from the above examples that the apparent deformation compensates for the Lorentz contraction only in the case of special characteristics of motion and special methods of observation and that, in general, the interpretation of the observed effect as a rotation is not correct.

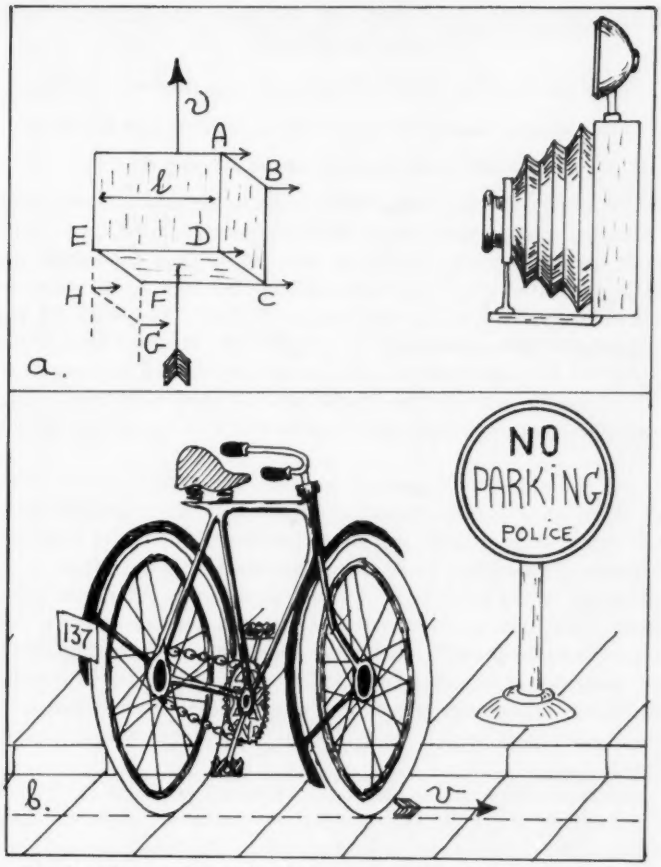

FIG. 1.—Photographing (*a*) a fast-moving cube and (*b*) a fast-moving bicycle (minus the rider).

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*THE THERMOSTATIC CONTROL OF HUMAN METABOLIC HEAT PRODUCTION**

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NAVAL MEDICAL RESEARCH INSTITUTE AND NATIONAL CANCER INSTITUTE

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The fact of human thermal homeostasis in warm or cold environments, at rest or under maximal activity and heat production, has stimulated numerous attempts to clarify its various aspects. Although such efforts have been made since 1885 or earlier, no integrated set of experimental facts has been forthcoming to explain it in terms of measurable effects and causes. Effects—responses by augmented metabolic heat production, sweating, or vasodilation—have not been demonstrated until recently[1] to be quantitatively and inseparably related to causes: stimuli of temperature at specific terminal receptor sites of our nervous system. Until such a causal analysis is accomplished, the homeostatic system cannot be considered clarified.

Lately, the introduction of gradient calorimetry[2, 3] (for measurements of the physiological responses) and cranial thermometry[4] (for measurements of the stimulus at the thermostatically controlled interior of the body) have sufficiently raised the power of resolution, which the instruments must have to solve their task. Moreover, simple ways have been devised to separate cutaneous from central temperatures. Thereby, true correlations between a response and either one of the two stimulant receptor temperatures under consideration are readily distinguished from false, coincidental correlations. (False correlations between a response and one of the two stimuli obtain as long as cutaneous and internal temperatures are themselves interrelated under physiological conditions without experimental interference.)

With these precautions against fallacious reasoning and with the refinements of methods as outlined above, an experimental resolution of "physical" heat regulation in man was first obtained and presented in these Proceedings[1] and elsewhere.[4, 5] (After these results were published, observations were extended to environments of 50° and 55°C and again no driving influences of skin thermoreception could be found. By cooling the skin below the threshold of cold receptor action, to 32°C (and less), however, it was possible to reduce the rate of sweating. It is as yet unknown whether this influence upon sweat glands is direct, or a consequence of vasoconstriction, or perhaps induced by cold reception.) Subsequently, the resolution of the combined "physical" and "chemical" mechanisms was carried out as follows.

Results.—In Figure 1, as a result of three months of experimentation with one healthy young man, rates of metabolic heat production in response to cold, and rates of sweating heat loss in response to heat, are plotted against cranial internal temperatures, measured at the tympanic membrane of the ear. Cold-stimulation was obtained in water baths; warm-stimulation, in the gradient calorimeter at high environmental temperatures. Coincidental correlations were deliberately disrupted through measures driving skin and central temperatures apart. Nevertheless, the deviations hardly exceeded the experimental errors and the plot is mean-

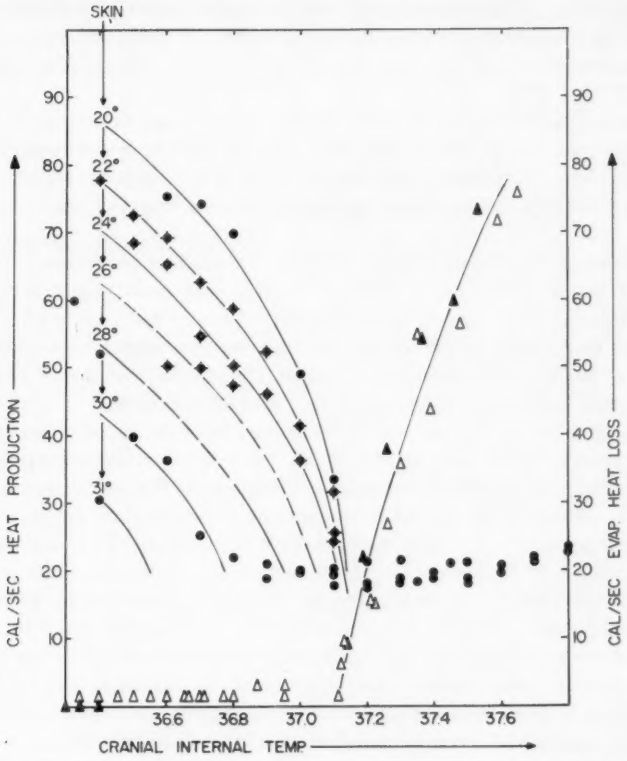

FIG. 1.—Experimental Resolution of "Chemical" and "Physical" Temperature Regulation. Thermoregulatory heat production (abscissae, circles, cal/sec) at low and constant cranial internal temperature (ordinates) is determined by steady cold-stimulation of skin (temperatures 31° to 20°C). Central warm-stimulation (ordinates) leads to depressing counteraction, which becomes complete at individual "setpoint of thermostat," 37.1°C. Thermoregulatory sweating (triangles, abscissae) is uniquely determined by internal sensory warm-reception. It begins at setpoint, rises to comparable evaporative loss (cal/sec). Result is tenacious maintenance of setpoint (homeostasis) over fourfold range of production or losses. *Note:* For narrow temperature range on ordinate, resolving power of classical methods was insufficient.

ingful on either side of a sharply determined "setpoint of the human thermostat" (at 37.11°C internal cranial temperature for this subject). The tenacious maintenance of a "setpoint temperature"—that is, the fact of human thermal homeostasis—is quantitatively explained as follows.

In response to minute changes with increasing *central* temperature, evaporative heat loss by sweating rises to extraordinary magnitude on the right side of the setpoint. Here the strict relation with cranial internal temperature is unaffected by the variations of *skin* temperature between 32° and 37°C that had been artificially introduced. On the left side, metabolic heat production rises to comparable values as internal temperature is reduced below the setpoint in minute changes on

this narrow scale. The magnitude of that particular response, however, is clearly dependent on whatever skin temperature prevailed, as indicated by the lines connecting measurements taken at any individual one of the various skin temperatures between 31° and 20°C.

It followed that "chemical" heat regulation by increased metabolic heat production was determined by both peripheral (skin) as well as central temperature, or thermoreception. It followed also that in "physical" temperature regulation by sweat-gland activity, *central* thermoreception was the origin of the driving warm-impulses.

In other words, in chemical heat regulation (controlling production), the central component acted as a synaptic station relaying and modifying afferent sensory impulses from the skin. By contrast, in physical heat regulation (controlling loss), the central component acted like a terminal receptor organ with first neurons, by showing the unique capability of translating minute changes of temperature, as stimuli, into nervous activity mediating powerful responses.

This puzzling contradiction will be explained later in our discussion with the existence, proven by classical authors, of two "centers" with fundamentally different characteristics. Additional experimental evidence shall first be introduced concerning the nature of the neural cold-messages from the skin, which Figure 1 so clearly demonstrates. By rapid and continuous recording of human oxygen consumption with new methods[6, 7] we have been able to show in various independent ways that the sensory messages driving thermoregulatory heat production in response to cold are a result of nerve impulses of increasing frequency from thermoreceptive nerve endings at the skin as investigated by Hensel and Zottermann.[8]

1. By transient application of the stimulus of cold to the skin in a water bath, in the absence of measurable changes of internal cranial temperature, the metabolic rate of man may be deliberately raised to, and lowered from, a rate of 3 to 4 times basal oxygen consumption.

2. To sudden lowering of total skin temperature, the rate of oxygen consumption responds with a transitory, overshooting "spike" and settles at a new and constant higher rate, commensurate with the new and lower temperature level. The frequency of electrical discharges from isolated thermoreceptive fibers of the cat in experiments of Hensel and Zottermann[8] responded in the same manner.

3. To sudden elevation of skin temperature, the metabolic rate of man responds with an overshooting and transitory depression of thermoregulatory heat production. The frequency of electrical discharges from isolated thermoreceptive fibers of the cat responded likewise.[8]

4. When the effects of changes in skin temperature are excluded, and only such measurements of metabolic rate and steady skin temperature are considered at which the cranial internal temperature was the same, for example 36.6°C, as in Figure 2*a*, a reproducible, peculiar relationship appears: metabolic rate first rises and then falls when skin temperature is brought below a critical level of maximal thermoreceptive response. The frequency of electrical discharges from isolated thermoreceptive fibers in ref. 8 followed the same pattern (Fig. 2*b*).

The extended series of experiments, from which only those performed at one certain internal cranial temperature, 36.6°C, had been selected for Figure 2*a*, is fully shown in Figure 2*c*. In this representation, the effect of skin temperature

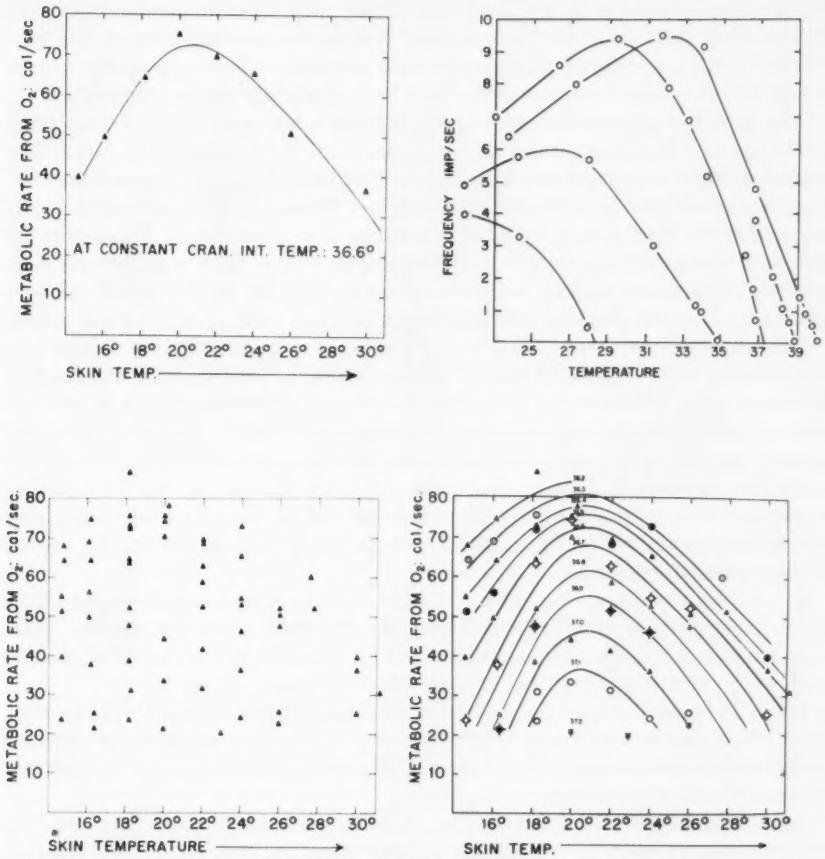

FIG. 2.—Experimental Resolution of "Chemical" Heat Regulation. (Skin-plot.)
(*a*) Metabolic response to steady cold-stimulation of skin (observed at *constant* central temperature, 36.6°C) attains maximum and then declines.
(*b*) Electrical discharge frequency from isolated thermoreceptive fibers in cat's tongue (after Hensel and Zottermann[8]).
(*c*) Metabolic rates plotted against skin-temperatures at *varied* cranial internal temperature yield seemingly senseless plot.
(*d*) Interconnection of measurements obtained at any one of many cranial internal temperatures in Figure 2*c* with "best-lines" yields Figure 2*d*, a family of curves, the resolution of "chemical" heat regulation. Parameters discussed in text.

upon the rate of oxygen consumption appears to be wholly obscured by another factor so that a senseless plot results.

This factor is the influence of cranial internal temperature, which had been deliberately and arbitrarily varied in the series of measurements. For when, as in Figure 2*a*, all measurements obtained at one and the same internal cranial temperature were interconnected with a common "best line," the senseless field of scattered points was converted into a family of curves and a complete and quantitative resolution of "chemical" heat regulation was obtained for this individual (Fig. 2*d*). This representation is different from, though equivalent to, the one in Figure 1,

which is based on the same observations. Either one of the two plots permits us to predict what the rate of thermoregulatory heat production would be in this individual for any combination of internal cranial and cutaneous temperatures. From Figure 2*d* some important parameters may be read without further explanation.

The threshold of skin thermoreceptor excitation was near 33°C (extrapolated to the right). Maximum excitation took place at 20–21°C skin temperature. The largest possible response was approximately 350 per cent of resting metabolic rate. Response-sensitivity between the threshold and the maximum was roughly 1 cal/sec heat production to each 0.2°C of skin temperature reduction. The depressing action of rising cranial internal temperature was of the order 1 cal/sec for each 0.01°C. Depression became complete between 37.2° and 37.0°C of central temperature, i.e. at the setpoint of this individual for the onset of sweat-gland action.

Interpretation.—A thermostatic neural mechanism, as described by Figure 1 in quantitative terms of stimuli and responses, cannot be anatomically organized in arbitrary ways. It must be based upon a certain minimum number of effector, central, and receptor components, interconnected as shown in Figure 3. (For the present purpose of interpretation, the background drawing of the brain in Figure 3 should be disregarded. While the calorimetric analysis demonstrates the existence of the components drawn with bold lines, the actual location of corresponding centers and pathways will be discussed only later with reference to classical discoveries of experimental surgery.)

a. A "heat *maintenance* center," *P*, receives from cold-receptive nerve endings of the skin through an afferent pathway, *sk*, afferent impulses for thermoregulatory heat production, which is initiated and maintained through an efferent pathway, *m*, in the metabolizing tissues of the effector organs.

b. A "heat *loss* center," *A*, without receiving warm-impulses from the skin, must translate the stimulus of temperature in its own cells into nervous impulses which initiate and maintain the responses of sweating and peripheral vasodilation through the efferent pathways *sw* and *v*.

c. Through a third efferent pathway, *d*, the depression of thermoregulatory heat production must be effected by sensory impulses from an internal receptor site, such as *A*, through connections with effector neurons of the "heat maintenance center," *P*.

In summary, center *P* functions simply as a synaptic relay station with one efferent and two afferent pathways. Center *A* functions for temperature like any other terminal receptor organ with first neurons for whatever quantity it "senses." Center *A* is active by way of three efferent pathways upon the three unconscious thermoregulatory functions.

This type of system satisfies the experimental facts thus far revealed by calorimetry and experimental surgery, while it does not invoke any speculative assumptions. It cannot, of course, rule out conclusively such speculative assumptions as have been occasionally entertained; for example, the existence of additional central thermoreceptive organs having efferent pathways, afferent to the effector neurons for sweating and vasodilation. Such organs have been assumed by some to exist elsewhere in the brain or in the large central vessels or even in the cranial mucous membranes where our measurements were carried out. There is, however, one hypothesis based on serious experimental observations: the possible existence of an

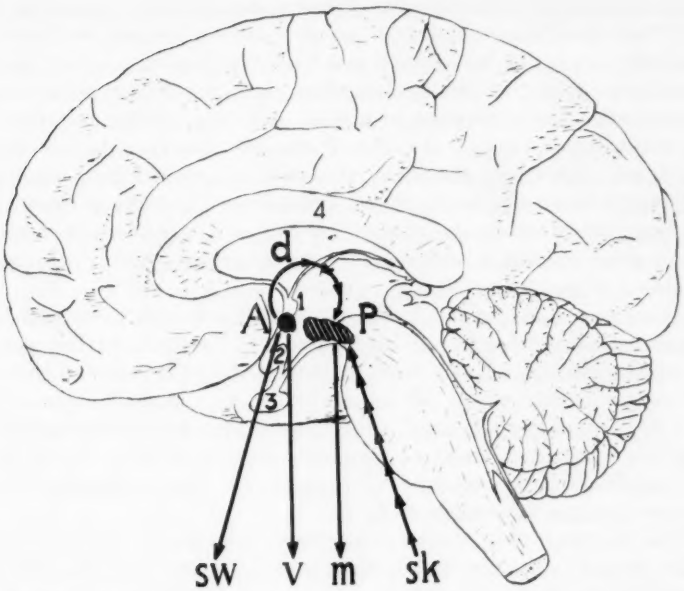

FIG. 3.—Minimum number, interconnections, and characteristics of thermoregulatory centers to be postulated from calorimetric evidence on man are shown with bold lines. Anatomical location of centers established by classical experimental neurosurgery on animals is shown by superimposition upon independent background drawing. Centers as shown have the characteristics required for the following functions: In the first place, sweat glands and cutaneous vessels (through efferent pathways *sw* and *v*) are operated from an *internal*-sensory receptor site, *D*. Secondly, metabolizing tissues (through pathway *m*) are operated for chemical heat regulation from *skin* as terminal receptor site (with afferent pathway, *sk*). Thirdly, metabolic response to increased frequency of cold-impulses in afferent pathway, *sk*, is counteracted and depressed by warm-impulses through another afferent pathway, *d*, originating from an *internal* thermoreceptive site such as *D*. Identity of one center, *D*, as the origin of both (depression of metabolic responses and operation of *sw* and *v*) is documented by references 17 and 18. So is *thermal indifference of center P*. Correct localization on background drawing, of centers *D* and *P* in anterior and posterior hypothalamus is documented by reference 9–17, 21–27, 29–35, and 38. Pathway *d* is anatomically identified by Hemingway,[33] upper part of pathway *sk*, by Anderson and Berry,[9] and *m* by Hemingway,[13–11] and Keller,[11] Anatomical "landmarks": (1) Commissura anterior, (2) Chiasma opticum, (3) Hypophysis, (4) Corpus callosum.

additional mechanism based on cold-receptor cells in the septum pellucidum.[38, 39] This mechanism might perhaps be active in deep hypothermia and support to a certain degree the major mechanisms described above.

Discussion.—Whereas the mere existence of a minimum of components, as shown in Figure 3, appears to be established with the calorimetric evidence of Figure 1, only experimental neuroanatomy or neurosurgery can ultimately localize these components within our central and peripheral nervous system. Such evidence in fact exists already and shall be discussed as follows:

1. The afferent pathway *sk* from the skin has been demonstrated by Anderson and Berry[9] who found in cats, after transection of the thoracic spinal cord, degenerating fibers from the lateral spinothalamic (Gowers') tract ascending into

and terminating within the subthalamus and posterolateral hypothalamic regions.

2. A "heat maintenance center," P, was delineated in the *posterior* hypothalamic gray as early as 1912 by Isenschmidt and Krehl[10] in experiments with ablation of dispensable tissue, and in 1914 by Isenschmidt and Schnitzler[11] by determination of the minimum lesion required to abolish shivering. Keller and Hare[12] have shown that after destruction of center P the heat loss functions (of center A) remain intact. Electrophysiologically, the function of center P has been established with electrical stimulation by Birzis and Hemingway[13] and the efferent pathway, m, has been determined by the same author with lesions,[14] electrical stimulation,[13] and with action-current recordings on shivering dogs.[15] Keller[16] has shown the continuation of pathway m in the pyramidal bundles.

3. Most important, the *indifference of center P to thermal stimulation* has been demonstrated independently by Hemingway *et al.*[17] with a diathermy electrode at the basal surface of the brain *near* the center, and by Freeman and Davis[18] using a diathermy electrode *within* the center. With the combined evidence of calorimetry and neurosurgery, center P in the posterior hypothalamus is therefore identified as a *synaptic* center relaying peripheral afferent impulses and incapable of responding itself to the stimulus of temperature. These characteristics differ fundamentally from those of center A.

4. The existence of a "heat loss center," A, in the *anterior* hypothalamus, was discovered by Aronsohn and Sachs in 1885.[19] It was delineated with improving precision by Barbour,[20] Ranson and Magoun,[21] Ranson,[22] Magoun *et al.*,[23] Hess and Stoll,[24] Folkow *et al.*,[25] and Anderson *et al.*[26] Ranson and Teague[27] have shown that its destruction leaves the "heat maintenance" functions intact, or even excessively responding (Pinkston, Bard, and Rioch[28]). Destruction of center A *eliminates* heat loss responses (Clark, Magoun, and Ranson[29]). Electrical stimulation of A *elicits* heat loss responses, causes the cessation of shivering (Hemingway *et al.*[30]), and thereby produces deep hypothermia (Andersson and Persson[31]). On patients, hyperthermia has been observed by Gagel[32] when center A was destroyed by neoplasm. While details in identification of pathways *sw* and *r* seem to require more extensive study, their existence is generally accepted.

5. Efferent pathway d, responsible for the central depression and thereby the control of shivering when temperature at center A increases, has been directly established by Hemingway[33] with the recording of action currents during electrical stimulation of the "heat loss center," A.

6. Most important, *thermal* (not only electrical) stimulation of center A has been shown by Magoun *et al.*[23] and by Stroem[34] to produce the heat loss responses. This phenomenon and, in addition, the cessation of shivering upon warm-stimulation of center A was shown by Hemingway[17] and by Freeman and Davis[18] independently. These authors applied the stimulus of heat selectively to center A while minimizing its effect upon center P, to find the origin of shivering depression.

7. Ultimately, Curt von Euler[35] has observed slow action potentials, commensurate with the artificially applied stimulus of temperature at center A, indicative, as he observes, of thermoreceptor cells. These findings are comparable to those of Svaetichin and MacNichol[36] who recorded slow potentials commensurate with the stimulus of light, near the optical receptor structures, first neurons of the retina. Another pertinent example are slow potentials commensurate with CO_2 tension and

respiratory volume in the respiratory centers of the medulla recorded by von Euler and Soederberg.[37]

In summary the neurosurgical evidence as quoted and the calorimetric evidence reported here are mutually consistent and confirmatory. They tend to establish the characteristics of a terminal sensory organ (concerned with warm-reception) for the anterior hypothalamic "heat loss center" designated "A" in Figure 3.

To understand the fact of human thermal homeostasis on the basis of the findings (Fig. 1) it remains to discuss briefly why conscious sensations and willful thermoregulatory actions of man must first provide the proper preconditioning. Too often the external conditions or workloads would demand a rate of heat loss so excessive that even the maxima of sweating and peripheral blood flow could not meet it. Also the air may be too humid to permit complete evaporation of the sweat-gland product. A cool environment may force internal temperature below the setpoint. These extremes, under which the system described in Figure 1 could not possibly function, are avoided by "Pavlovian" action. For one example at skin temperatures of 31°C the gap between the desired setpoint, 37.1°C (where oxygen consumption *should* begin to rise) and the intersect of the 31°C line at which it actually would (36.6°C), is bridged by the Pavlovian measure of voluntary muscular exertion. Iberall[40] has found that people warm themselves by deliberate movements whenever skin temperature drops below 34–33°C. Similarly, in other environments, conscious sensations of temperature will lead to voluntary locomotion into a more comfortable environment, to application or removal of clothing, to changes of bodily posture and radiating surface, to immersion in warm or cool water, to voluntary exercise, to building a shelter against cold or for shade, and ultimately to artificial heating or cooling with external sources of energy. Thus, autonomic and Pavlovian measures are equally important in their own ways; the first in providing for the range of human temperature control, the latter for its almost unbelievable precision.

In view of constructive comments on conclusions drawn in references 1, 4, 5, and here, the authors wish to state that they do not disregard the existence of additional thermoregulatory activity at segmental levels nor certain direct influences of skin temperature upon sweat glands or vessels. In the vascular system those additional factors were observed with our methods, and discussed in references 1 and 4. In the sudomotor system and in shivering they are perhaps too small to be detected as long as the powerful hypothalamic system is in operation.

Summary.—By cranial thermometry combined with indirect and direct calorimetry, the two mechanisms of human "chemical" and "physical" temperature regulation have been experimentally resolved in terms of metabolic, sudomotor, and vasomotor responses of reproducible magnitude to the degree of cold-stimulation at thermoreceptive nerve endings of the skin and warm-stimulation of an extremely sensitive, centrally located thermoreceptive organ. The simplest possible neuroanatomical basis for explanation of the facts observed has been discussed with reference to the findings of classical experimental neurosurgery. It is concluded that the function of the "heat maintenance center" in the posterior hypothalamus is synaptic, relaying activity of increasing frequency from thermoreceptive endings in the skin for increasing response by metabolic heat production. This center is indifferent to the stimulus of temperature. The "heat loss center" in the anterior

hypothalamus, however, acts as a terminal receptor organ for temperature, comparable to the retina, the anatomically related receptor organ for light. This organ controls not only the direct responses to heat by vasodilatation and sweating but also through a third efferent pathway, afferent to the "heat maintenance center," it indirectly controls, with counteracting impulses, the response by metabolic heat production to cold-stimulation of the skin. The result of this threefold function is a thermostatic performance of astonishing power and precision.

Navy Hospital Corpsmen D. L. Drake and F. D. Crabill and, on several occasions, Ensign C. Chestnut or one of the authors volunteered for the extended experiments with exposure or immersion. Corpsman R. D. Arrieta underwent one exceptionally rigorous test. The expert technical assistance of Mr. G. W. Newlon and Mr. L. R. Younkins is gratefully acknowledged.

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*COCHLEAR POTENTIALS IN THE MARMOSET**

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From a comparative standpoint the primate ear claims a special interest, for this order is the one to which man belongs. It is more than a little surprising, therefore, that our knowledge about the hearing of members of this group is severely limited. Apart from man himself, only the chimpanzee and Old World monkeys (the rhesus monkey and its close relatives) have been tested for auditory acuity.

These considerations led Seiden,[1] working in our laboratory, to carry out auditory measurements in the marmoset, one of the New World primates that is considered more primitive than the species mentioned above. He used five animals of the species *Hapale jacchus* and trained them to respond to sounds by a shock avoidance method. The animal was placed in a tipping cage and was required to cross over its center line when a tone was sounded, being given a mild electric shock if it failed to do so within 0.5 to 3 seconds after the tone began. Observations of threshold sensitivity were attempted over a frequency range from 100–80,000 cycles, but all the animals reached their limit between 25,000 and 37,000 cycles.

After these measurements had been completed, the same animals were used for cochlear potential studies, and the results of these studies are the subject of the present report.

The animals were anesthetized with diallylbarbituric acid and ethyl carbamate (Dial), in a dosage of 1 cc/kg of body weight. The recording electrode was a platinum foil on the round window membrane, with an indifferent electrode in inactive tissue nearby. In all the animals the observations were made on the right ear. The stimulating and recording equipment described earlier in our experiments on the cat[2] was used to obtain measurements over the range of 100–100,000 cycles.

Some of the results are given by the dashed-line curves of Figures 1 and 2, together with Seiden's curves of threshold acuity shown by solid lines. Figure 1 shows a representative ear, and the data for three other animals were closely similar to these. As will be seen, the potentials were obtained over the full range of 100–100,000 cycles. As the frequency was raised above 100 cycles, the sensitivity in terms of these potentials improved slowly, except for an irregularity around 700 cycles, until 1,500 cycles was reached, after which the level remained fairly uniform up to 20,000 cycles. Beyond this point the sensitivity was poorer, and the course of the curve was somewhat more irregular than elsewhere.

A comparison with the behavioral threshold curve shows a number of differences. The gain in sensitivity as the frequency is raised above 100 cycles is noticeably

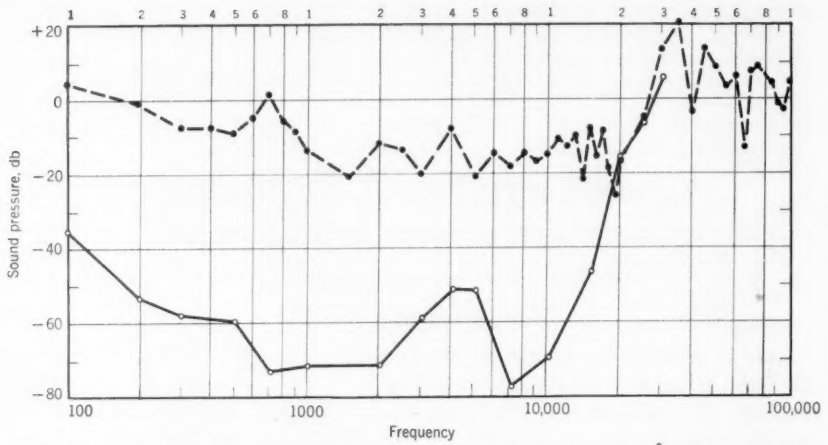

FIG. 1.—Auditory sensitivity in a marmoset as determined by behavioral and cochlear response methods. The ordinate shows the sound pressure in decibels relative to 1 dyne/sq cm at the threshold of hearing for the two ears (solid line) and that necessary to produce a potential of 1 microvolt in the right ear (dashed line).

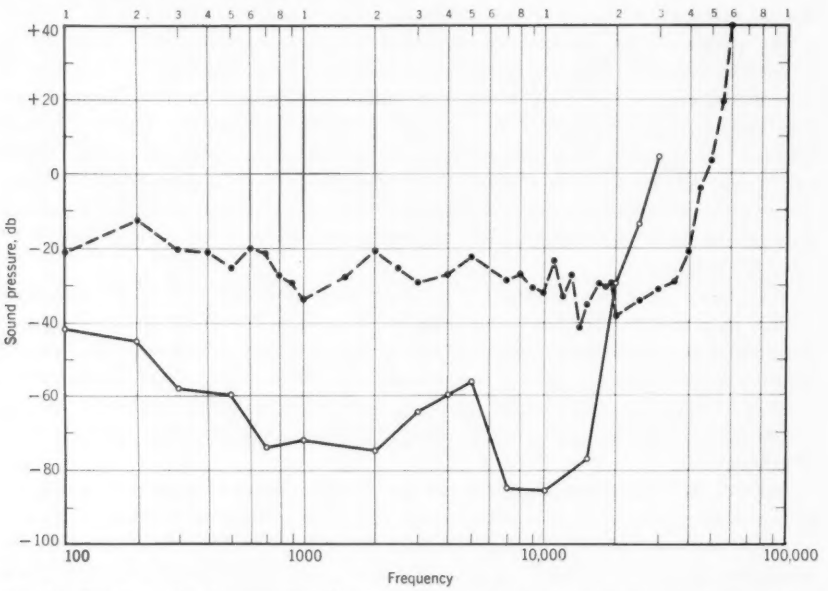

FIG. 2.—Results as in the preceding figure for a second animal with relatively poor cochlear potential sensitivity for the highest tones.

more rapid for the behavioral function. There is also a striking variation in the curve between 2,000 and 7,000 cycles, which consists of a fall in sensitivity to 5,000 cycles, then a rapid rise, and finally a rapid fall. The curve continues to show this fall in sensitivity to its end at 30,000 cycles, which represents the upper limit of hearing under the conditions of the experiment.

Every one of Seiden's animals exhibited the variation between 2,000 and 7,000 cycles and in exactly this same region of frequency. It is notable that this feature has no counterpart in the cochlear potentials. It evidently has its origin in the auditory nervous system.

Figure 2 represents an ear that does not conform exactly to the others of the group. This animal was a female, and from her general appearance she was judged to be older than the others. A few days before the cochlear potential measurements were made she gave birth to two young. The threshold acuity was much the same as for the first animal, but a difference appeared in the cochlear potential curve in the high frequencies. Beyond 30,000 cycles the response required increasingly intense sounds, and could no longer be obtained beyond 60,000 cycles.

An explanation has already been offered[3] for the attainment of an upper limit by the acuity function while the cochlear potentials are still maintained. It is supposed that certain mechanical and neural factors operate so as to enhance the acuity to high tones, thereby accounting for the greater slope of this function and the remarkable degree of sensitivity exhibited in the region of 6,000–15,000 cycles. However, these factors ultimately reach a limit of effectiveness and then decline. The most likely factors that operate in this fashion are the following. The pattern of response on the basilar membrane becomes narrower as the frequency rises, and at the same time there is a shift of the region of most vigorous activity toward the basal end of the cochlea. The basal shift finally moves the main response area to a region of the cochlea where the density of innervation falls off precipitously. Further, the excitability of nerve fibers by the cochlear potentials increases with frequency over a considerable range, but at last attains a limit. Finally, at low and intermediate frequencies there is a synchronous relation between sound waves and auditory nerve impulses, and therefore the rate of impulses rises with the frequency. In the upper frequencies, however, the synchronous relation is disturbed and finally fails, and the average rate of impulses suffers a decline. For all these reasons, we believe, the acuity and cochlear potential curves vary in their courses in the high frequencies and have different limits.

* These experiments were aided by a contract with the Office of Naval Research and by Higgins funds allotted to Princeton University.

[1] Seiden, Herbert R., "Auditory Acuity of the Marmoset Monkey, *Hapale jacchus*," Thesis, Princeton University, 1957.

[2] Wever, E. G., J. A. Vernon, W. E. Rahm, and W. F. Strother, "Cochlear Potentials in the Cat in Response to High-Frequency Sounds," these PROCEEDINGS, **44**, 1087–1090 (1958).

[3] Wever, E. G., "The Cochlear Potentials and Their Relation to Hearing," *Ann. Otol. Rhinol. & Laryngol.*, **68**, 975–989 (1959).

ERRATA

In the paper entitled "A Bacteriophage Containing RNA," by Tim Loeb and Norton D. Zinder, which appeared in volume 47, number 3, the first sentence at the top of page 289 should be corrected to read as follows: "The molar ratios were 1.17 ± 0.03, 1.00, 1.21 ± 0.06, and 1.13 ± 0.07, respectively."

In the paper entitled, "Comparison of Theoretical with Observed Values of the Periods of Free Oscillation of the Earth," by C. L. Pekeris, Z. Alterman, and H. Jarosch, which appeared in volume 47, number 1, pages 91–98, the authors wish to make the following corrections:

Page 96, equation (9), for $\sigma_1 = \sigma_0 + \tau_+ \omega$, read $\sigma_1 = \sigma_0 - \tau_+ \omega$.

In the paper entitled "Population Studies in Predominantly Self-Pollinated Species, I. Evidence for Heterozygote Advantage in a Closed Population of Barley," by S. K. Jain and R. W. Allard, which appeared in volume 46, number 10, pages 1371–1377, the authors wish to make the following correction:

On page 1375, the top three lines should read as follows:

$$\left.\begin{aligned} P_{n+1} &\propto x\{s(P_n + {}^1/_4R_n) + t(P_n + {}^1/_2R_n)^2\} = x\{P_n + {}^1/_4(sR_n - te_n)\} \\ Q_{n+1} &\propto y\{s(Q_n + {}^1/_4R_n) + t(Q_n + {}^1/_2R_n)^2\} = y\{Q_n + {}^1/_4(sR_n - te_n)\} \\ R_{n+1} &\propto {}^1/_2sR_n + 2t(P_n + {}^1/_2R_n)(Q_n + {}^1/_2R_n) = R_n - {}^1/_2(sR_n - te_n) \end{aligned}\right\} \quad (1)$$

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